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Methods for Toxicity Tests of Single Substances and Liquid Complex Wastes with Marine Unicellular Algae

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This manual describes methods for laboratory toxicity testing with marine unicellular algae. Principles of aquatic toxicity testing with algae are summarized. Methods for maintenance of algal cultures, choice of test species, estimation of population density, detection of living and dead cells, expression of toxicant effect, and bioaccumulation of toxicants are discussed.

Further, terms related to algal toxicity tests, equipment needed for algal toxicity tests, and methods for preparation of algal growth medium are defined. When population growth tests are combined with studies on bioaccumulation and algicidal or algistatic properties of a toxicant, an overview of important effects is obtained, and several relevant effects criteria provide a base for regulation of single substances and mixtures of substances.

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Please note that Figure 3 (p. 28) and Figure 6 (p. 41) are transposed. The figure captions are correct.

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March 1988

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Gerald E. Walsh

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Table of Contents

		Page
Abstract		v
Acknowledgments		vi
1. Principles of Aquatic Toxicity Testing with Algae		1
1.1	Introduction	1
1.2	Growth Curve of Unicellular Algae in Culture	1
1.3	Factors That Influence Growth of Unicellular Algae in Culture	3
1.4	Maintenance of Algal Cultures	7
1.5	Choice of Species	7
1.6	Measurement of Population Density	8
1.7	Living and Dead Cells	9
1.8	Numerical Expression of Effect	9
1.9	Addition of Toxicant	10
1.10	Bioaccumulation	10
1.11	Summary	10
1.12	References	11
2. Toxicity Tests With Marine Unicellular Algae		16
2.1	Introduction	16
2.2	Definitions of Terms	16
2.3	Personnel	17
2.4	Safety	17
2.5	Disposal of Toxicants	18
2.6	Quality Control	18
2.7	Chemical and Physical Properties of Test Substances	18
2.8	Chemical Analyses of Test Substances	19
2.9	Equipment	19
2.10	Test Species	21
2.11	Axenic Culture	21
2.12	Growth Medium	22
2.13	Medium for Use in Studies on Toxicity of Heavy Metals	23
2.14	Medium for Use with Tests on Liquid Complex Mixtures	25
2.15	Fractionation Procedure for Liquid Complex Wastes	29
2.16	Test for Living and Dead Cells	29
2.17	Bioaccumulation	29
2.18	Documentation and Reporting of Results	29
2.19	References	29
3. Growth Test with Single Substances		31
3.1	Preparation of Toxicant	31
3.2	Toxicant Concentrations	31
3.3	Test Procedure	32
4. Growth Tests with Liquid Complex Wastes		38
4.1	Collection of Waste	38

4.2 Filtering of Waste 38
4.3 Sterile Techniques 38
4.4 Estimation of Population Density 38
4.5 Calculation of Effect Concentration 40
4.6 Procedure for Effects of Whole Waste 42
4.6.1 Preparation of Medium 42
4.6.2 Procedural Steps 42
4.7 Procedures for Effects of Inorganic and Organic Fractions . 44
4.7.1 Preparation and Testing of Inorganic and Organic Fractions 44
4.7.2 Procedural Steps 46
4.8 Procedures for Effects of Inorganic and Organic Subfractions 49
4.8.1 Inorganic Subfractions 49
4.8.1.1 Preparation and Testing of the Cation Subfraction 50
4.8.1.2 Preparation and Testing of the Anion Subfraction 51
4.8.2 Organic Subfractions 53
4.8.2.1 Preparation and Testing of the Subfractions .. 53
4.9 Procedure for Extraction and Testing of Particles 55
4.9.1 Extraction of Organics 55
4.9.2 Testing of Extract 55
4.10 References 55
5. Bioaccumulation Test 60
6. Enumeration of Living and Dead Cells 63
6.1 References 64

Abstract

This manual describes methods for laboratory toxicity testing with marine unicellular algae. It consists of six parts: Part I describes principles of aquatic toxicity testing with algae. It describes algal growth curves and factors that influence algal growth including light, temperature, composition of culture medium, and pH. Methods for maintenance of algal cultures, choice of species for testing, estimation of population density, detection of living and dead cells, expression of toxicant effect, and bioaccumulation are discussed.

Part II defines terms related to algal toxicity testing, describes equipment needed for algal toxicity tests, and gives detailed methods for preparation of algal growth medium and estimation of population density by cell counts and spectroscopy.

Part III describes the recommended toxicity test with single substances. The test is conducted for 48 h, at which time population densities of control and treated cultures are measured and the median effect concentration and growth rates are calculated.

Part IV gives methods for estimating effects of liquid complex wastes on growth of algae. Methods are described for analysis of whole waste, its organic and inorganic fractions, anion, cation, base-neutral, and acid subfractions, and particulate matter.

Part V describes a method for estimation of bioaccumulation of single substances by algae in culture.

Part VI gives a method for distinguishing living and dead algal cells.

Each of the above tests addresses a different aspect of hazard evaluation of single substances or mixed waste with regard to inhibition or stimulation of algal population growth, survival, and possible introduction of toxic substances into food chains. When population growth tests are combined with studies on bioaccumulation and algicidal or algistatic properties of a toxicant, an overview of important effects is obtained, and several relevant effects criteria provide a base for regulation of single substances and mixtures of substances.

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1. Principles of Aquatic Toxicity Testing with Algae

1.1 Introduction

This manual describes methods for use of marine unicellular algae in toxicity tests. Although no common method has been used by testing laboratories, those described here have been used successfully in tests with pesticides, heavy metals, other toxic substances, and complex industrial wastes. Methods have differed mainly in composition of algal growth medium, test species, and endpoint, each of which may affect expression of toxicity. The methods described here recommend a carefully formulated growth medium, sensitive algal species, and three means for estimation of algal population density.

Toxicity testing with unicellular algae requires application of the principles of phycology and microbiology to culturing, handling, and exposing the organisms. Success in culturing and testing depends upon the knowledge and competence of the responsible person. The technician who works with unicellular algae must be familiar with methods for maintenance of nonbacterized cultures and their transfer to fresh media, identification of algal species and deviations from normal morphology, rates of growth of algal species in culture, use of laboratory instruments, such as microscopes, counting chambers, analytical balances, light and fluorescence spectrophotometers, and all other aspects of culture and testing described below.

1.2 Growth Curve of Unicellular Algae in Culture

Algal population growth in culture follows a predictable course under controlled conditions of light, temperature, and nutrient concentration. After inoculation of growth medium with a known number of cells of a standard age, the usual pattern of population growth includes a lag phase, phase of exponential growth, phase of declining growth rate, stationary phase, and death phase (Fig. 1). See Kaufmann (1981) for a discussion of use of growth curves. The period of time for each phase under optimal conditions is specific for each algal species. It is not necessary to use synchronous cultures in toxicity tests because the exponential growth phase, in which cell number increases at a high rate, is required.

The pattern of population growth is very important in toxicity testing because toxicants and growth stimulating substances cause deviations from normal (Fig. 2). Generally, two responses of algal populations to test substances are measured: (1) Rate of growth over part or all of the exponential phase is determined and growth of treated cultures is compared with growth of control cultures. Population density or a mathematical

Figure 1. Pattern of population growth of algal cultures: 1. lag phase; 2. exponential growth phase; 3. phase of declining growth rate; 4. stationary phase; 5 death phase (after Fogg, 1965).

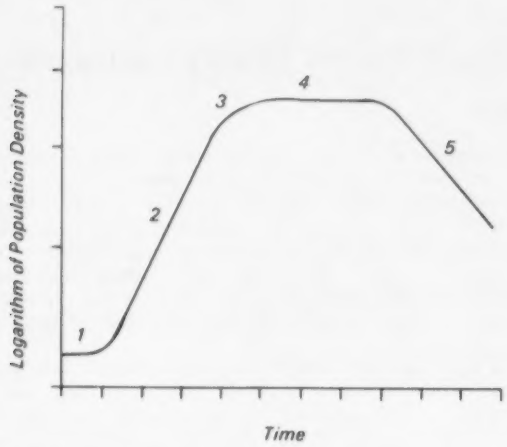
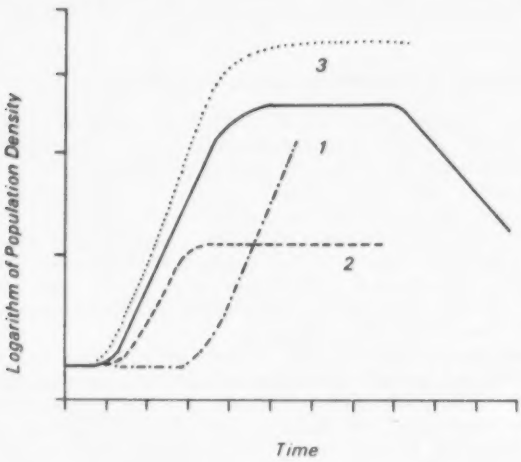


Figure 2. Deviations of algal growth from the normal growth curve: 1. increased lag phase; 2. reduced growth rate and yield; 3. enhanced growth rate and yield.



expression of the growth rates of control and exposed cultures at a selected time may be compared. (2) Final population density is an important criterion for detection of growth-inhibiting and growth-stimulating substances and for characterizing eutrophic natural waters.

Length of the lag phase may be critical to success because it can determine duration of the toxicity test. To minimize the length of the lag phase, cultures must be initiated with the proper number of cells from algal stocks in the early exponential phase. Use of older cells may cause a lengthy lag phase.

Duration of the toxicity test is very important. Algal tests should be conducted within as short a time period as possible to minimize problems with fate of the test chemical in the exposure flasks. When the solution, with algae, is placed under lighting required for algal growth, concentration of the chemical may decline due to photodegradation, molecular instability, adsorption to vessel walls, volatilization from the surface of the medium, adsorption to algal cells, absorption by algal cells, or biodegradation. Shape of the growth curve in response to a chemical may be due, at least in part, to behavior of that chemical in the test system. For example, loss of chemical over a selected period of time could determine the rate of decrease in growth or period of increased lag phase.

Because of the confounding factor of chemical fate, it may be difficult to interpret algal toxicity data from static tests. Walsh (1983) demonstrated that the IC50 (interpolated concentration of a toxicant that would inhibit algal population growth by 50%) increased with time of exposure. Thus, it is recommended that test duration be no longer than three days, at which time population densities (which are low) and growth rates (which are high) of control and exposed populations may be compared by either graphical interpolation from density data or by comparison of the growth rates.

If the final yield of algae is required for growth stimulation studies, the test must be continued until the early stationary phase.

1.3 Factors that Influence Growth of Unicellular Algae in Culture

There are five major factors that affect growth of algae in culture: light (quality, intensity, duration), temperature, and composition, salinity, and pH of the growth medium. Each must be controlled carefully, with little deviation among tests.

LIGHT Algae are cultured under artificial illumination in the laboratory, but such illumination may differ greatly from natural sunlight in quality (spectral composition), intensity, and duration. These three characteristics may be regulated for optimal growth of individual species. When species with different optimal requirements are held in a single growth chamber, average conditions that support growth can be maintained.

Intensity of light has a strong influence on growth and physiological state of algal populations (Beardall and Morris, 1976; Hitchcock, 1980; Perry et al., 1981; Cosper, 1982; Soeder and Stengel, 1974; Gallagher et al., 1984; Falkowski et al., 1985). Gallagher et al. (1984) reviewed a portion of the literature on effects of light intensity on rates of growth and photosynthesis, pigments, the photosynthetic unit, and means of photoadaptation of algae. In

addition, light intensity may influence release of products of photosynthesis from algal cells (Fogg, 1965; Fogg et al., 1965; Hellebust, 1965, 1967; Nalewajko, 1966; Watt, 1966, 1969; Bellin and Ronayne, 1968), thus increasing the amount of dissolved organic matter in growth medium and, perhaps, affecting uptake of the test substance.

Light intensity may also affect behavior of the test chemical. Rates of photodecomposition of numerous compounds are related to intensity of light and, when photodecomposition is rapid, it may be the major factor that determines toxicity. Kamp-Neilsen (1969) reported that toxicity of copper to *Chlorella pyrenoidosa* was related to light intensity.

Care must be taken to insure little variation in illumination of algal cultures because light intensity can influence growth rate and physiological characteristics of algal populations and behavior of test chemicals. Intensity of light in growth chambers must be kept constant for all tests and should not exceed the optimal for algal population growth. Light intensity should be recorded daily, and when it falls to 90% of optimal, the lamps should be replaced.

Spectral composition of light can affect growth and photosynthetic rates and cellular morphology of algae (Humphrey, 1983). As for other photosynthesizing plants, energy of visible light in the wavelength range of 400 to 729 nm is incorporated by pigments specialized for absorption of electromagnetic radiation. The various algal divisions contain different amounts of the three types of pigments that absorb light: (1) chlorophylls that absorb blue and red light, (2) carotenoids that absorb blue and green light, and (3) phycobilins that absorb green, yellow, and orange light. Tungsten lamps emit light in wavelengths used by algae, but also emit a large amount of heat that is difficult to control in growth chambers. Fluorescent lights also emit wavelengths that can be utilized by algae, and "cool white" fluorescent tubes are used commonly, sometimes in conjunction with tungsten lamps.

Spectral signatures of tungsten and fluorescent lamps can usually be obtained from manufacturers. Whatever the form of lighting, it is essential that lamps be replaced before gross changes occur in spectral signature. Replacement when intensity diminishes is probably adequate for consistent spectral output, but changes in color of light can sometimes be detected visually. If a color change is seen, the lamp should be replaced.

Marine unicellular algae may be cultivated and tested under continuous light or under dark-light cycles. Humphrey (1979) described photosynthetic characteristics of algae grown under both types of regimes: algae grown under a 12 h light - 12 h dark cycle had a higher photosynthetic rate and a higher photosynthesis:respiration ratio, but a lower growth rate than those grown under continuous lighting. These findings are important to culture and testing of algae. All stock algal cultures and all tests should be maintained under the same lighting conditions to insure comparability of results. In addition, although temperature change has little effect on photochemical processes, optimal temperature for population growth may vary with light intensity: increase in temperature increases the point at which light saturation occurs (Soeder and Stengel, 1974). The test recommended here requires continuous lighting at constant temperature to insure population densities large enough to allow statistical evaluation of toxicity data after two days.

TEMPERATURE Each algal species has a specific culture temperature for optimal growth at a given light intensity. Soeder and Stengel (1974) reviewed algal adaptation to and effects of temperature on population growth rate, cell growth, and metabolic processes. Algae adapt quickly to temperature change. However, if tests are to be done at a temperature other than that at which algal stocks are maintained, then new stocks must be prepared and adapted to the new temperature. Temperature should not vary by more than one degree C from the desired temperature.

ALGAL GROWTH MEDIA There have been numerous reviews of algal nutrition in relation to composition of growth medium (Provasoli et al., 1957; Krauss, 1958; Provasoli, 1958, 1966; McLachlan, 1959, 1964; Droop, 1961, 1969; Gerloff, 1963; Nicholas, 1963; Hunter and Provasoli, 1964; Kester et al., 1967; Venkataraman, 1969; Prat et al., 1972; Healy, 1973; Stein, 1973; O'Kelley, 1974). Droop (1969) gave 65 references to different growth media for marine algae. The major thrust in development of marine algal medium has been elucidation of nutritional requirements and amounts of nutrients and their ratios required for growth and normal cell morphology. Modern media are composed either of natural or artificial seawater to which nutrients are added. Nutrient additions include orthophosphate (PO_4^-), nitrate (NO_3^-), potassium, magnesium, calcium, sulphur, boron, chelated trace metals, vitamins, and silica (for diatoms and some chrysophytes and xanthophytes). See Droop (1977) for a discussion of nutrition of phytoplankton.

Media made from seawater have been used extensively for culture of unicellular algae. Guillard and Ryther (1962) described a medium (Medium f) made from natural seawater that has proved to be excellent for culture of many algal species. Medium f and its nutritional variations, f-1 (Guillard and Ryther, 1962) and f/2 (Stein 1973), are still in use today.

Although natural seawater-based media are often excellent for culturing algae, diel and seasonal variability in its composition may control rate of growth. Johnston (1962) demonstrated seasonal variability of ocean surface water to support growth of algae. Media prepared from surface water of the Gulf of Mexico support growth of algae in our laboratory between October and April, but not between May and September, even though nutrient enrichments are identical. Unless a relatively unchanging source is available, toxicity tests with medium prepared from natural seawater may not yield comparable data if the seawater is collected at different times.

Growth media prepared from pure chemicals and distilled or deionized water have been described (Morel et al., 1979; Harrison et al., 1980). See Morel et al. (1979) for a discussion of requirements for algal synthetic medium. Such media have the advantage of constant salt and nutrient composition without possible variations that can occur in natural water. This is especially important if fluorescence intensity is used for estimation of population density (Tunzi et al., 1974; Slovacek and Hannon, 1977; Gilbert et al., 1986). Synthetic media are particularly advantageous for toxicity tests because there is little batch-to-batch variation: the only significant variables are the toxicants and their concentrations. In our laboratory, the medium of Morel et al. (1979) produced good growth with normal cell morphology. However, medium prepared as described by Morel et al. (1979) is unacceptable in toxicity tests because algae are very sensitive to the toxicant

carrier, acetone. We have modified nutrient concentrations slightly to make it acceptable in toxicity tests.

Algal growth medium can also be prepared from commercial sea salt mixtures. Preparation of media from commercial sea salts is rapid and easy, and algae grow well in them when nutrients are added. Although overall composition is known, there may be lot-to-lot variability, with possible inhomogeneity within lots of commercial mixtures. We have compared growth and responses to toxicants in media made from five commercial sea salt mixtures and natural seawater (Walsh et al., in press). We have used one product, Rila Salts® (Rila Products, Teaneck, NJ), with good results in many tests. Although salinity was the same and only small differences were found in nutrient concentrations, differences in response to the same toxicant were large. Sensitivity to toxicants was generally greater in medium made from Rila Salts than other commercial formulations (Walsh et al., in press), with good growth and normal morphology in carrier controls.

Algal medium must be sterilized before use. Although autoclaving has been used by many investigators, it is not recommended because of possible contamination of medium from steam under pressure. It is recommended that basal medium be pasteurized and that nutrients be sterilized by membrane filtration and added to basal medium immediately before the test.

Responses of algae to acetone when the medium of Morel et al. (1979) is used demonstrate that care must be taken in choosing an algal medium; although good growth and normal morphology may be obtained with a medium, that medium may be unsuitable for use in toxicity tests.

When liquid complex wastes are to be tested, growth medium is prepared by addition of salts and nutrients used in synthetic medium (Walsh and Alexander, 1983; Walsh et al., 1980, 1982; Walsh and Garnas, 1983), and synthetic medium is used as the untreated control. Filtration should be done only if algae are present in the waste before testing. The medium cannot be sterilized because physical and chemical properties of the waste may be altered.

SALINITY Salinity of growth medium affects growth and primary production of algal populations (Braarud, 1951; Nakanishi and Monsi, 1965; Vosjan and Siezen, 1968). Algal species may be stenohaline, oligohaline, or euryhaline. Euryhaline species tolerate a wide salinity range by adjusting concentrations of organic solutes in the cell (Hellebust, 1976a, 1976b; Ahmed and Hellebust, 1985), and osmoregulation and osmoadaptation may also rely on activity and specificity of ionic pumps (Soeder and Stengel, 1974). Osmotolerant marine algae require time to adjust to hypertonic and hypotonic stress (Gilmour et al., 1982, 1984), and it is important that algae be cultured in the salinity of the toxicity test for several stock transfers before the test begins. Salinity must not change when inoculum culture is added to test medium.

pH Since growth media and natural seawater are generally around pH 8, this should not present a problem in culturing marine algae, but pH should be measured and recorded before each test. Measurement of pH after a test is of little value because algal activities may change it by as much as one pH unit even in the highly buffered medium. Flasks should be shaken gently and continuously to maintain gaseous equilibrium between growth medium and

air. This should insure sufficient carbon dioxide in the medium to maintain pH at less than 8.5.

Deviations from the normal pH can indicate improper preparation of medium. We have used media between pH 7.6 and 8.2 with good growth and normal morphology. It is best to use a single medium that is constant from test to test to avoid effects of pH on toxicity of test substances. However, when medium is prepared from highly acidic or basic liquid waste, rather than from deionized water, pH is adjusted to approximately 8.

1.4 Maintenance of Algal Cultures

Stock algal cultures are maintained in the laboratory under conditions of light, temperature, and growth medium identical to those of the toxicity test. Inoculation of new medium is done under aseptic conditions during the phase of declining growth or no later than early in the stationary phase to insure that healthy cells are transferred. Microscopic examination of stocks should be made for cellular morphology at each transfer. If abnormal cells are seen, transfers should be made at an earlier time in the growth curve, the culture may be contaminated, or the growth medium may have been prepared incorrectly. Usually, abnormal cells are caused by excess of one or more of the trace metal nutrients. Stock cultures are checked for aerobic and anaerobic bacterial contamination at approximately monthly intervals, or more frequently if contamination is suspected.

Algal stocks for toxicity tests should be in the early exponential growth phase to avoid transfer of extracellular algal products and because over 99% of the cells are living and healthy during this phase (Walsh and Alexander, 1980). The stocks should be examined for cellular morphology and only normal cells, as found during the exponential phase of population growth, should be used.

1.5 Choice of Species

The major factor that determines quantitative expression of toxicity is the species used in the toxicity test. Some species are more sensitive than others to toxicants, but it is impossible to predict which species, or clone of a species, will be most sensitive or resistant to a particular toxicant. Choice should be from ecologically important or abundant, generally sensitive species that can be cultured in the laboratory and with population growth rates that allow estimation of density within two or three days of inoculation. Bonin et al. (1986) listed six principal criteria for choice of test species: (1) wide geographical distribution, (2) well-known nutrient requirements, (3) good taxonomical characterization of the strains, (4) small genetic and phenotypic variability, (5) high growth rate, and (6) ease of handling. They also presented an important critical review of algae commonly used in toxicity tests.

When possible, test species should occur naturally in the areas that may be affected by the toxicant. For example, if a toxicant is a threat to an inshore environment, a species isolated from a nonpolluted inshore environment may be used for maximum utility of the test (Murphy and Belastock, 1980). Usually, algal clones must be purchased from a laboratory that maintains a culture collection.

It is usually impractical to maintain more than one or two test species in culture. Walsh and Alexander (1980) recommended the diatom, *Skeletonema costatum*, because it meets most criteria listed above and is generally sensitive to pollutants. However, estimation of cell numbers cannot be made on an electronic particle counter because the diatom forms long chains. Bonin et al. (1986) offered compelling arguments for use of the diatom, *Thalassiosira pseudonana*, which occurs as a single cell.

The diatom, *Minutocellus polymorphus* Hasle, von Stosch and Syvertsen, is recommended here. It is sensitive to most pollutants, population density can be estimated easily, and the period of exposure to toxicants is only 48 hours. In addition, estuarine and oceanic clones are available for site-specific studies.

1.6 Measurement of Population Density

There are three common methods for estimation of population density of marine algal cultures: cell counts, light spectroscopy, and fluorescence spectroscopy. Brezonik et al. (1975) reviewed methods for estimation of population density of cultured algae; Rehnberg et al. (1982) reviewed limitations of electronic particle counting methods when used in algal tests. Each method estimates a different aspect of the population. Cell counts give the number of cells in a volume of medium, and when performed under the microscope, cell morphology can be examined. Light spectroscopy estimates the concentration of suspended particles (algae) by absorption of light, and fluorescence spectroscopy estimates the amount of chlorophyll in cells in suspension. Cell counts and spectroscopy do not give information on cell size, morphology, physiological state, or if cells are living or dead.

No single method for population analysis is completely satisfactory. For example, a toxicant may cause a large number of small cells or a small number of large cells, relative to untreated control populations, with biomass equal to the control. Such effects lead to false conclusions from all three methods, unless results are also expressed as cell size, measured with a size distribution meter on an electronic particle counter, or as fluorescence per cell. Even here, counts may be misleading because particle counters cannot distinguish living from dead cells. Fluorescence results may be deceiving because fluorescence yield is a function of chlorophyll concentration and chloroplast shape. Toxicants may cause change in cell chlorophyll content without causing change in cell number, or they may cause deformation of thylakoids, thus affecting fluorescence yield.

Despite problems associated with estimation of population density, we have found good correlation between results from the three methods and, on a practical basis, recommend them for use with most toxicants. The researcher should be aware, however, that some toxicants, notably heavy metals, cause conformational changes in algal cells that may affect results, and that effects cannot be reported simply as mathematical expressions (IC50, EC50, SC20; see below) derived from cell counts or spectroscopal data. Enumeration of deformed cells may be vital for determination of effects of some substances.

1.7 Living and Dead Cells

It is often desirable to identify the mechanism that inhibits algal population growth. Toxicants may kill cells (algicidal) or simply inhibit the rate of reproduction (algistatic). Presumably, algicides would have greater effects than algistats on algae in natural waters because algicides kill resident and allochthonous species and reduce ability of populations to adapt to the pollution.

Crippen and Perrier (1974) described a method for identification of living and dead cells by direct counts with the vital stain, neutral red, and the mortal stain, Evans blue. Reynolds et al. (1978) used the method to demonstrate that chlorine-produced oxidants did not kill algae, and Walsh (1983) described use of Evans blue in toxicity tests with pesticides. In the method of Walsh (1983), algae are grown in uncontaminated medium for 48 h and the living and dead cells enumerated. The algae are then exposed to toxicant for 24 h, living and dead cells again enumerated, and decrease in growth rate and increase in number of dead cells compared to untreated control populations. The method provides for rapid estimation of algistatic and algicidal potentials of toxicants.

1.8 Numerical Expression of Effect

Bioactivity of compounds toward algae is expressed as the interpolated concentration that would cause a specific effect in a specific period of time. Thus, the 72-h IC50 is the calculated concentration that would inhibit population growth or a physiological process of treated cultures by 50% of untreated cultures in 72 h. The IC50 is also called the EC50 (effective concentration). Similar expressions are used when cell death is observed (lethal concentration, LC) or when growth or a physiological process is stimulated (stimulatory concentration, SC). Test durations have ranged from hours for physiological and growth endpoints to weeks for growth endpoints, and effects as percentages of untreated controls have been reported between 1 and 99. Effect concentrations are calculated from tests in which response to a graded series of toxicant concentrations is measured and percentage (of untreated cultures) response is related to concentration. Methods that have been used for calculation of algal response include: graphical interpolation (Yamane et al., 1984; APHA, 1985), moving average interpolation (Harris, 1959; Stephan, 1977), probit regression (Finney, 1971; Ordog, 1981), and binomial (Harris, 1959). Walsh et al. (1987) discussed these methods as applied to growth tests, compared EC50s calculated by them, found no significant differences, and suggested use of graphical interpolation because it is simple, rapid, and accurate.

Two other expressions are used to describe toxicity. The Lowest Observed Effect Concentration (LOEC) is the lowest concentration of a substance in a toxicity test that has an adverse effect on the exposed population when compared to the untreated control population. The No Observed Effect Concentration (NOEC) is the highest concentration used in a test that has no observed effect on the exposed population.

1.9 Addition of Toxicant

In toxicity tests, a range of toxicant concentrations is used to encompass the desired percentage response, e.g., if the IC50 is to be used for expression of algal response, concentrations should include the NOEC, the 100% response concentration, and concentrations that inhibit growth by more than and less than 50%, such as 35 and 65%. At least five concentrations should be used for establishment of the concentration-response curve.

Most toxicants are poorly soluble in water and must be dissolved in a solvent carrier before addition to test medium. Concentration of solvent is critical to success of a test. It must be as low as possible and the same for every toxicant concentration. Carrier controls must be included in every test, with algal growth equal to that of noncarrier controls. We have found (unpublished data) that acetone is less toxic to marine algae than two other frequently used carriers, triethylene glycol and dimethyl sulfoxide. However, acetone can affect results by interaction with toxicants, and the publications of Stratton (Stratton et al., 1980, 1982; Stratton and Corke, 1981a, 1981b; Stratton, 1986, 1987) should be consulted for review of this subject.

1.10 Bioaccumulation

Although not necessarily related to toxicity, accumulation of toxicants from sublethal concentrations in water may be important to toxicant fate and transfer through food chains. Rice and Sikka (1973), Hollister et al. (1975) and Walsh et al. (1977) showed that living and dead algal cells can accumulate toxicants. Bahner et al. (1977) demonstrated transfer of the pesticide, Kepone, from contaminated algae to oysters. Bioaccumulation studies are recommended for a more complete understanding of relationships between algae and toxicants.

1.11 Summary

Algal toxicity tests include numerous microbiological procedures and culture criteria that must be controlled carefully for accuracy and reproducibility. There must be test-to-test consistency in technique to insure comparable data. Algal stock cultures must be maintained in a healthy, growing state. Constant monitoring of light intensity and temperature, care in preparation of growth medium, analysis of growth medium for salinity and pH, and morphological examination of algal stock, inoculum, and control cultures should insure healthy populations with reproducible growth rates. Algal populations used as inocula for toxicity tests must be taken from the early exponential phase of growth in all tests. By carefully following established good laboratory practices for culturing and testing, the trained technician can generate data that describe potential effects of chemical substances on population growth and survival and on bioaccumulation by marine unicellular algae.

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2. Toxicity Tests with Marine Unicellular Algae

2.1 Introduction

Marine unicellular algae (phytoplankton, microalgae) are essential to normal function of marine (estuarine and oceanic) ecosystems. As the main primary producers, algae form the first link in food webs, oxygenate the water, and are important in cycling of dissolved organic and inorganic substances. It is important, therefore, to be able to predict possible effects of chemical pollutants on algal populations with data from toxicity tests on sensitive species.

The methods given here are static laboratory toxicity tests for detecting effects of single compounds and complex wastes on growth of laboratory algal populations, death of algal cells, and determination of bioaccumulation by algae *in vitro*. Although specific algal species are recommended, the method may be used with other species, either unmodified or with slight variations in light intensity, temperature, or salinity in accordance with their requirements. The test is not designed to detect fate of toxicants. Effects on growth and survival in relation to initial concentration of parent compound are expressed, but effects of possible degradation products, behavior of chemicals in the test system, or effects upon physical or chemical characteristics of the growth medium are not.

2.2 Definition of Terms

Unicellular Algae -- Members of the Kingdom Plantae whose individuals are composed of a single cell and whose growth form may be unicells, chains, or groups. Those used in toxicity tests are phytoplankton and are known as microalgae in contrast to the multicellular macroalgae.

Axenic Culture -- Culture whose living components are known. In axenic algal cultures, algal species and bacterial contaminants have been identified. Generally, axenic algal cultures consist of a single species without bacterial contamination.

Growth Medium -- The liquid in which algae are cultured. It consists of a basic salt component in water that is similar in composition to seawater and to which nutrients are added. It may also be made from natural seawater.

Population Growth -- Increase in number or weight (biomass) of algal cells in a laboratory population. Cell size or morphology may change during rapid population growth.

Growth Rate -- Change in cell number or biomass of a population over a specific period of time. This is generally expressed as a positive integer, but may be negative if a toxicant causes death and disintegration of cells during the testing period.

Exponential Growth Phase -- Time of population growth *in vitro* at which cell number increases exponentially.

Population Density -- Number of cells in, or weight of, an algal population at a specific time. In algal toxicity tests, population density is estimated at the beginning of a test and at one or more times during the period of exponential growth to allow calculation of the growth rate.

Inoculum Culture -- Stock culture of algae in the exponential phase that is diluted to a specific concentration of cells and added to test medium.

Untreated Control Culture -- Algal culture that is an integral part of every toxicity test. It is a culture that is prepared, incubated and analyzed as all other cultures in the test, except toxicant and its carrier are not added.

Carrier Control Culture -- Algal culture that is an integral part of every toxicity test in which a solvent carrier is used. It is a culture that is prepared, incubated, and analyzed as all others in the test, except it contains the same volume of solvent carrier as treated cultures, but without the toxicant.

Rangefinding Test (Screening Test) -- Test conducted to determine concentrations of a toxicant to be used in the definitive test.

Definitive Test -- Test conducted to define the toxic or stimulating concentration of a chemical. It yields data for calculation of the IC50, LC50, or SC20.

IC50 (EC50) -- Interpolated or calculated concentration of a toxicant that would inhibit population growth or any other biological process of algae by 50% compared to the controls in a specific period of time. This has also been called the RC50 (concentration that reduces growth by 50%). The term RC50 is not recommended here because it is an erroneous expression of effect. Toxicity causes a lower growth rate in relation to control growth: it does not reduce growth rate in treated cultures.

LC50 -- Interpolated or calculated concentration of a toxicant that would kill 50% of the cells in a specific period of time.

SC20 -- Interpolated or calculated concentration of a substance that would cause population density of a treated culture to be 20% greater than the controls in a specific period of time.

NOEC -- No observed effect concentration. This is the highest concentration of a substance that had no observed effect in a toxicity test.

LOEC -- Lowest observed effect concentration. This is the lowest concentration of a substance that caused an observed effect in a toxicity test.

2.3 Personnel

Personnel designated to perform toxicity tests with algae must be trained in the principles and techniques of phycology and microbiology. Successful maintenance of axenic cultures, sterile transfer, identification of deviations from normal morphology, early detection of problems, and numerous other aspects of algal culture and testing can be accomplished only by scrupulous attention to details by knowledgeable people.

2.4 Safety

Safety of laboratory personnel must be insured before initiation of algal tests with toxic substances and solvent carriers. Manufacturer's data sheets

and other sources of information on effects of the test material on human health must be consulted and proper safety measures applied before testing. Care is especially important when working with liquid complex wastes, where contents of the mixtures may be unknown. Operators must wear protective clothing (laboratory coats, gloves, respirators) whenever exposure may occur to skin or by inhalation, suitable absorbants for carrier and toxicant spills must be at hand, and the laboratory must be well-ventilated. All liquids must be transferred by automatic pipette, never by mouth, and flame or sparks must not be present when solvents are used. Stock solutions must be stored in a refrigerator equipped with a spark-free motor. Laboratory coats, gloves, and respirators must be worn when cleaning contaminated glassware with solvent.

When algal tests are done for EPA, each laboratory must have an approved, detailed, written safety plan that describes methods for safe handling of toxicants and solvents.

2.5 Disposal of Toxicants

The testing laboratory must have a written plan, approved by EPA, for safe disposal of toxicant stocks and stock dilutions in solvent, contaminated growth medium with algae, solvent used for rinsing glassware, and contaminated equipment such as pipettes, laboratory coats, gloves, and absorbant used to clean up spills. Disposal will generally require employment of a commercial company licensed by the State for hauling and disposing of toxic waste according to RCRA regulations.

2.6 Quality Control

The best quality control that can be applied to algal toxicity tests is related to competent personnel, close adherence to all aspects of the method as described, and consistency in day-to-day operations. Careful quality control must be applied to maintenance of algal stock cultures, control of light intensity and temperature, rigorous cleaning of glassware, a draft-free working area, application of microbiological techniques for maintenance of sterility, and periodic testing for bacterial contamination of stocks.

When algal tests are done for EPA, each laboratory must have a detailed, written quality control plan that describes requirements for culturing and testing of algae. A laboratory notebook that records data showing adherence to good laboratory practices required by EPA should be available for inspection by the Project Officer.

2.7 Chemical and Physical Properties of Test Substances

Chemical and physical properties of toxic substances and solvent carriers should be known before the test begins. They can often be obtained from the manufacturer, seller, or handbooks on such properties, and are used for setting toxicant concentrations and interpretation of results. Information is required on solubility, volatility, vapor pressure, rate of decomposition in water, and octanol-water partition coefficient.

2.8 Chemical Analyses of Test Substances

Chemical analyses must be performed to confirm identity and purity of the toxicant and to determine percentage recovery in growth medium before initiation of algal tests. Quantitative analyses must also be done on medium that contains the concentrations of toxicant used in each test. Identification and quantitation of major organics, anions, cations, PO_4^-, and NO_3^- in complex mixtures is also desirable, and results of chemical analyses should be submitted with test results. When algal tests are done for EPA, the testing laboratory must have an approved, detailed, written set of protocols for chemical analyses, including a plan for quality control.

2.9 Equipment

GLASSWARE All glassware must be of borosilicate glass or equivalent. Glassware used for stock cultures and in toxicity tests should be capped with stainless steel closures or foam plugs. Flasks and closures distributed by Bellco Glass Co., Vineland, NJ, work well. Foam plugs may be used only once because they absorb volatile chemicals. Their use is discouraged because they must be disposed of as solid waste.

Volume of growth medium used for maintenance of algal stocks should be limited to an amount easily handled for sterile transfer. It is suggested that 200 ml of growth medium in a 500 ml volume Erlenmeyer flask is a convenient system because sterile transfer is difficult with larger flasks. Volume of growth medium should not exceed one-half that of the Erlenmeyer flask, whatever the volume.

Flasks of 125 ml volume are used in toxicity tests. If heavy metals are to be tested, the flask should be coated with silicone film such as General Electric SC-87 dry film (Pierce Chemical Co., Rockford, IL) or equivalent. The method for silicone coating was described by Davey et al. (1970). Use 2.5 - 5.0% SC-87 in cyclohexane to coat the flasks. Swirl the solution to cover the flask wall, dry in air, cure at 150 - 175 C for 4 h, rinse with deionized or glass-distilled water, and dry again. Inspect all flasks after each test and recoat over the old silicone layer if necessary.

Fernback flasks of 2.8 L volume are used in bioaccumulation studies.

It is necessary to use clean flasks that are not scratched inside because such flasks cause precipitation of salts from growth medium. Medium in all flasks should be examined for precipitated salts before each test.

Glassware must be washed thoroughly to avoid carryover of toxicant from test to test. When a test is completed, dispense all contaminated algal cultures to a safe container and rinse three times with acetone into the container. Rinse the flasks with tap water and soak them overnight in 1N HCl, scrub in phosphate-free detergent, rinse 10 times with tap water and three times with glass-distilled or deionized water, dry in an oven, and store capped in a draft- and dust-free room.

GROWTH CHAMBERS Separate growth chambers must be maintained for algal stock cultures and toxicity tests. They must be kept under identical, carefully controlled conditions of light (quality and intensity) and temperature. Light should be from cool white fluorescent tubes, with variable control of

light-dark cycles or continuous lighting. Intensity may vary with algal species, but is generally between 50 and 150 $\mu E\ m^{-2}\ sec^{-1}$. Intensity may be controlled by rheostat or by placing cultures at selected distances from the fluorescent tubes. Light intensity in the tests recommended here may be between 100 and 125 $\mu E\ m^{-2}\ sec^{-1}$ (590-735 ft can; 6,350-7,900 lux) at the surface of the growth medium. When light intensity falls below 100 $\mu E\ m^{-2}$ sec-1, the fluorescent tubes should be replaced. Intensity of light from new tubes falls rapidly during the first four days of use and remains constant for many days thereafter. Therefore, it is best if tests are not initiated until four days after the fluorescent tubes are replaced.

Temperature in the growth chamber may also vary with requirements of individual algal species. Generally, a chamber that can maintain constant temperature between 10 and 25 C ($\pm$0.5 C) is adequate. Temperature in the test described below is 20 C.

LIGHT METER Light intensity must be monitored at least once a day at the level of the surface of the growth medium. The light meter must be calibrated against a standard, and measurements must be recorded daily.

TEMPERATURE MONITOR A device for continuous recording of temperature must be installed in the growth chamber. It should read in °C, and all original temperature records should be kept in a permanent file.

MICROSCOPE A microscope with resolving power that allows detailed examination of algal cells is needed for checking cell morphology. It must have a mechanical stage that accepts a hemacytometer for counting cells.

STERILIZATION OVEN Algal growth medium is sterilized by heat. The sterilization oven must be large enough to contain all flasks of each toxicity test and must maintain its temperature at 60 C for 4 h.

AUTOCLAVE An autoclave is used to sterilize everything other than growth medium. The autoclave must have a drying cycle to insure dry glassware and filters.

PIPETTORS Medium for toxicity tests is added to exposure flasks with a 50-ml volume pipettor. The Ace Glass (Louisville, KY) Volumetril Bulb and 500-ml volume flask work well. Nutrients are added with Eppendorf pipettes (or equivalent) fitted with disposable tips. Metal and vitamin additions require sterile tips. Sterile, disposable glass pipettes are required for culture transfers and sampling.

HEMACYTOMETER A hemacytometer is needed to estimate population density of inoculum cultures and for enumeration of cells in toxicity tests.

HAND TALLEY A multi-channel hand talley device is required for counting cells on a hemacytometer and for differential counts of living and dead cells.

ELECTRONIC CELL COUNTER An electronic particle counter may be used to estimate population density of algae that occur as unicells, but not as doublets, chains, clumps, etc. It is desirable to have an attachment to the counter for estimation of average cell volume.

SALINOMETER A hand-held salinometer is required to check salinity of growth medium and raw liquid waste.

VISIBLE LIGHT SPECTROPHOTOMETER Population density may be estimated with a visible light spectrophotometer equipped with variable wavelength control and a 10-cm light path. This equipment may be used

when the only particles in the culture are algal cells, the population is in the exponential phase of growth, and the medium is not highly colored.

FLUORESCENCE SPECTROPHOTOMETER Population density may also be estimated by fluorescence spectroscopy of intact cells. This method is very sensitive, especially at low population density.

ROTARY SHAKERS Cultures in algal toxicity tests must be shaken gently to insure exchange of oxygen and carbon dioxide between the atmosphere and growth medium, and to maintain the cells in suspension for optimal utilization of nutrients and light.

pH METER pHs of stock and test media are measured and recorded before each transfer or test.

CENTRIFUGE A high-speed centrifuge is used in studies on bioaccumulation. Buckets may be up to 500 ml in volume and lined with Teflon (E.I. DuPont de Nemours, Newtown, CT) or equivalent, but never metal.

MAGNETIC STIRRER Magnetic stirrers are used for preparation of growth medium and resin for cation removal.

2.10 Test Species

Algal species used in toxicity tests may vary in accordance with requirements and objectives of the tests, and the method given here should accommodate most marine unicellular algae after slight modifications of salinity or nutrients.

If a specific species is not required, the test species should be *Minutocellus polymorphus* Hasle, von Stosch and Syvertsen (1983). This species, earlier called *Bellerochea polymorpha* by Hargraves and Guillard (1974), is found in estuaries and the open ocean, is easily cultured, and is sensitive to toxicants (Fisher et al., 1972; Fisher, 1977). In the United States, *M. polymorpha* can be obtained from the Bigelow Laboratory for Ocean Studies, West Boothbay Harbor, Maine 04755. It is suggested that Clones 675D, BCN, or SD be used in toxicity tests because they are oceanic and thus tend to be more sensitive to most toxicants than clones from inshore waters. However, Clone SAY 7, isolated from an estuary, may also be used.

An important consideration for use of *M. polymorphus* is its rapid growth in culture. Toxicity tests can be completed after 48 h growth, thus reducing the influence of photodecomposition, volatilization, and adsorption of toxicant that may occur in tests of longer duration.

Starr (1971) and Rosowski and Parker (1982) have identified laboratories from which algal cultures may be obtained.

2.11 Axenic Culture

Axenic cultures of algae are uncontaminated by bacteria or other organisms. Algal cultures used in toxicity tests should be axenic and tested for the presence of bacteria at approximately monthly intervals.

The ATCC Media Handbook (1984) gives formulations of media for detection of bacterial contamination. At least two media should be used:

1.Bacto Peptone	10.0 g
Succinic Acid	1.0 g
$(NH_4)_2SO_4$	1.0 g

$MgSO_4 \cdot 7H_2O$	1.0 g
$FeCl_3 \cdot 6H_2O$	2.0 g
$MnSO_4 \cdot H_2O$	2.0 g
Synthetic Seawater	1.0 L
2. Bacto-Tryptone Yeast Extract	5.0 g
Glycerol	3.0 g
Synthetic Seawater	750 ml
Distilled or Deionized Water	250 ml

Synthetic Seawater

NaCl	27.5 g
$MgCl_2$	5.0 g
$MnSO_4 \cdot 7H_2O$	2.0 g
$CaCl_2$	0.5 g
KCl	1.0 g
$FeSO_4$	1.0 g
Distilled or Deionized Water	1.0 L
Adjust to pH 6.8 with KOH	

Dispense 10 ml of medium into test tubes, cap with stainless steel closures or foam plugs, and sterilize by autoclaving at 121 C for 15 min. Inoculate the sterile media with 0.25 ml of stock algal culture and incubate at 20 C. Examine the media for bacterial growth daily for seven days. After seven days, streak aliquots of the media on 1.5% agar prepared with the bacterial media and incubate at 20 C for seven days.

If bacterial contamination is detected, the stock culture must be purified or a new culture obtained. Techniques for purification are given by Stein (1975). The easiest method is by treatment with antibiotics, and the techniques described by Stein (1975) for purification in liquid and on agar should be used.

2.12 Growth Medium

BASIC MEDIUM The algal growth medium is a modification of that described by Morel et al. (1979). This medium, called "Aquil," is completely artificial and chemically defined. It is designed to reduce contamination by trace metals and to contain nutrient concentrations similar to those of natural seawater, thus precluding complications due to precipitation of salts. Morel et al. (1979) recommended removal of cations from the major salt and nutrient solutions by use of an ion exchange column. This step may be used when the toxicants in question are heavy metals, but it is possible that the ion exchange process may modify the medium and introduce contaminants. Untreated medium can be used with all other toxicants. Separate algal stocks should be maintained in both media.

Formulations of salt and nutrient solutions are given in Table 1. The sequence of steps in preparation and inoculation of 1 L of growth medium is:

1. Dissolve all salts, except $MgCl_2 \cdot 6H_2O$, in approximately 750 ml of glass-distilled or deionized water in a 1-L volumetric flask. The water should have a resistivity of at least 18 megohm-cm at 25 C.
2. When all of the salts have dissolved, add the $MgCL_2 \cdot 6H_2O$.

3. When the $MgCl_2 \cdot 6H_2O$ has dissolved, add 0.25 ml of each of the PO_4^-, NO_3^-, and SiO_3^- nutrient solutions.

4. Bring to 1 L volume with glass-distilled or deionized water. Salinity should be approximately 35 parts per thousand. Check salinity with a salinometer.

5. Filter the growth medium through a 0.45 µm porosity filter washed previously with 1 L of deionized or distilled water.

6. Dispense into culture flasks. Add approximately 200 ml to each 500 ml volume Erlenmeyer flask for stock cultures. Add 50 ml to each 125 ml volume Erlenmeyer flask with a volumetric bulb. Dispense approximately 1 L into each Fernbach flask for bioaccumulation studies.

7. Heat sterilize in an oven at 60 C for 4 h.

8. Immediately before the test, mix equal volumes of the metal and vitamin solution, sterilize by filtration, and add 2 ml to each stock culture flask with 200 ml growth medium, 0.25 ml to each test flask with 50 ml growth medium, and 5 ml to each liter of medium in bioaccumulation studies.

9. Use a sterile membrane filter (0.45 µm porosity) and non-metallic filter holder affixed to a sterile syringe for sterilization of the metal and vitamin mixture. Filter into a sterile capped tube and add to growth medium with an Eppendorf pipette equipped with a sterile tip.

10. For toxicity tests, add toxicant in no more than 0.025 ml solvent carrier to medium in exposure flasks; add the same volume to medium in carrier control flasks. All flasks must receive the same amount of solvent carrier. Reserve other flasks without carrier to serve as untreated controls.

11. Add 1 or 2 ml of old stock at the end of the exponential growth phase to 200 ml fresh medium for maintenance of algal stocks. For toxicity tests, add 1 ml of inoculum culture, prepared as described below, to 50 ml growth medium. These transfers must be done by microbiological methods that insure sterility.

2.13 Medium for Use in Studies on Toxicity of Heavy Metals

Contaminating cations may be removed from the basic salt and nutrient solutions before tests for heavy metal toxicity in water. The method for cation removal was given by Morel et al. (1979). Their report should be consulted before proceeding with this test, which should never be used with acetone carrier. A chromatography column, packed with resin that retains cations by chelation, is used to strip impurities before the salts and nutrients are mixed. For one column:

1. Use Chelex 100 resin (drymesh 100-200, Na form; Bio-Rad Laboratories, Richmond, CA).

2. Rinse 15 g resin for 5 min in approximately 200 ml methanol in a 400-ml beaker.

3. Rinse the resin three times in glass-distilled or deionized water.

4. Add 300 ml of the solution to be cleaned and, while stirring on a magnetic stirrer, titrate very slowly to pH 8 (± 0.1) with 1N NaOH for the salt solution and 1N HCl for PO_4^-, NO_3^-, and SiO_3^- solutions. Titrate until pH is stable for at least 30 min.

5. Fill a 10 x 250 mm glass chromatography column with the solution to be cleaned. A column with a reservoir at the top will save much time. While

Table 1. Composition of Salts and Nutrients to be Used in Culturing and Testing Marine Unicellular Algae (Morel et al., 1979)

Substance[1]	g L^{-1}
NaCl	24.53
$CaCl_2 \cdot 2H_2O$	1.54
KBr	0.10
NaF	0.003
KCl	0.70
H_3BO_3	0.03
Na_2SO_4	4.09
$NaHCO_3$	0.20
$SrCl_2 \cdot 6H_2O$	0.017
$MgCl_2 \cdot 6H_2O$[2]	11.1

[1] The first nine substances are weighed previously to the test. Several batches of dry salts may be prepared at one time and stored in tightly capped glass containers for use when needed. Since it is convenient to prepare two liters of medium for each test, batches of salts that contain twice the amounts given here may be stored and used to prepare growth medium in glass-distilled or deionized water.

[2] The $MgCl_2 \cdot 6H_2O$ is dried at 104 C, stored in a desiccator, and added only after the other salts have dissolved.

Nutrients

1.Dissolve 1.38 g $Na_2HPO_4 \cdot H_2O$ and 5.26 g NaCl in 1 L glass-distilled or deionized water.

2.Dissolve 8.5 g $NaNO_3$ in 1 L glass-distilled or deionized water . Adjust pH to 8.0 with 1N NaOH.

3.Dissolve 3.55 g $Na_2SiO_3 \cdot 9H_2O$ and 4.38 g NaCl in 1 L glass-distilled or deionized water. Adjust pH to 8.0 with 1N HCl.

Trace Metals

1.Dissolve 0.47 g Na_2EDTA and 0.031 g $FeCl_3 \cdot 6H_2O$ in approximately 300 ml glass-distilled or deionized water in a 500 ml volumetric flask. If heavy metals or liquid waste are to be tested, use one-half of the iron concentration and prepare this solution without EDTA immediately before the test

2.Dissolve 0.249 g $CuSO_4 \cdot 5H_2O$ in 1 L glass-distilled or deionized water.[1]

3.Dissolve 0.265 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 0.595 g $CoCl_2 \cdot 6H_2O$ in 1 L glass-distilled or deionized water.[1]

4.Dissolve 0.455 g $MnCl_2 \cdot 4H_2O$ and 0.115 g $ZnSO_4 \cdot 7H_2O$ in 1 L glass-distilled or deionized water.[1]

the solution is dripping at about 40 ml min^{-1}, pour the resin slurry that was

5.Add 0.25 ml of 2, 3, and 4 to 1 above and take to volume. This if the metals working mix.

[1] Unused portions of these solutions may be frozen for up to a year for later use.

Vitamins

1.Dissolve 110 mg Vitamin B_{12} in 100 ml glass-distilled or deionized water.

2.Dissolve 10 mg biotin in 100 ml glass-distilled or deionized water.

3.Add 0.025 ml Vitamin B_{12} and 0.25 ml biotin solutions to approximately 75 ml glass-distilled or deionized water in a 100 ml volumetric flask. Dissolve 0.005 g thiamin hydrochloride in it and take to volume. This is the vitamin working mix. Keep frozen when not in use.

adjusted to pH 8 into it at a constant rate and without interruption to insure a continuous, homogeneous column of resin.

6. After all of the slurry has been added, continue flow of salt or nutrient solution until the column is completely packed.

7. Pass the solution to be cleaned through the column at the drip rate of 5 ml min^{-1}.

8. After it has passed through the column, bubble air vigorously through the solution to return the pH to 8. The air should be filtered through a cotton plug.

9. Prepare separate columns as above for the salt, PO_4^-, NO_3^-, and SiO_3^- solutions. The metal and vitamin solutions are not stripped of cations.

10. After the pH is returned to 8, medium is prepared as described above for nonstripped medium.

11. Resin should be replaced after 50 L of salt solution or 100 L of nutrient solution have eluted. It should also be replaced if it becomes discolored.

12. The columns may be kept at room temperature when not in use, but they must not become dry. If the resin breaks, or if any part of it dries out, it must be regenerated to pH 8 and replaced.

It is suggested that several columns for stripping the salt solution be used simultaneously because this step is time-consuming.

2.14 Medium for Use with Tests on Liquid Complex Mixtures

Follow instructions for preparation of medium as described above, except use liquid waste instead of water in the salt solution. Do not filter the waste before adding salts unless algae are present, and do not sterilize. Flasks used in waste studies should be coated with silicone as described above. Polycarbonate flasks may also be used. Prepare the metal solutions (Table 1) without EDTA.

Liquid waste must be collected, stored, and shipped in unreactive containers at 4 C and tested as soon as possible after receipt at the laboratory. If the waste cannot be analyzed immediately, store it in the dark at approximately 4 C. Characteristics such as pH, salinity, color, smell, and presence of solids should be reported with results of toxicity tests.

ESTIMATION OF POPULATION DENSITY Three methods for estimation of population density are acceptable: cell counts, light spectroscopy, and fluorescence spectroscopy.

1.Cell counts -- Estimation of cell numbers may be made by counts on a hemacytometer. A sample is taken with a sterile pipette after vigorous swirling of the culture and added to both sides of the hemacytometer. All cells in the four corner and one central squares of each chamber are counted with a multi-channel hand tally. The total is the number of cells in a cubic millimeter of culture medium. This method has the advantage of visual inspection of algal cells, and those of abnormal morphology may be noted during the counting procedure. It is, however, time-consuming, relatively imprecise, and considerable precision is lost as cell numbers decrease. Photomicrography of hemacytometer preparations with subsequent image analysis is acceptable for estimation of population density.

Cells may be enumerated on an electronic particle counter. This method is more rapid and precise than the hemacytometer method, but it can be used only with species that occur as unicells.

Minutocellus polymorphus occurs as unicells in cultures up to 3 days old. Chain formation sometimes occurs in older cultures, and electronic particle counters cannot be used to estimate population density. Since the test described here requires only two days, chain formation is of little importance.

If an electronic particle counter is used, growth medium, metal mix and vitamin mix should be filtered through a 0.22 µm porosity filter before sterilizing, and an uninoculated blank sample of medium prepared for use in instrument calibration with regard to background particle interference. This method is used best with a particle size distribution meter to determine influence of toxicant concentration on cell size. All cultures should be examined microscopically for abnormal cells when counts are made on an electronic particle counter. If appreciable numbers of abnormal cells are present, estimate their percentage by a differential count of 100 cells. In order to determine the IC50 as described below, calculate the average counts for control, solvent carrier control, and each treatment culture.

2.Light spectroscopy -- Light spectroscopy is a rapid, efficient way to estimate population density, provided that the only particles in suspension in growth medium are algal cells. The spectrophotometer must be calibrated to zero with growth medium before each test, and checked again for maintenance of zero calibration after the series of absorbance readings is completed. The spectrophotometer should be equipped with a recorder for producing a printed direct readout of results. Keep the printed readout in the laboratory notebook. Absorbance of the cultures must be measured across a 10-cm light path to insure precision at low population density and at a wavelength specific for the algal test species. This may be determined by scanning between wavelengths of 500 to 700 nm and choosing the wavelength that produces the highest optical density with a culture of algae. The wavelength is approximately 680 nm for *M. polymorphus*. Examine the cultures microscopically for abnormal cellular morphology. If appreciable numbers of abnormal cells are present, estimate their percentage by differential counts of 100 cells. In order to determine the IC50 as described

below, calculate the average optical density for each control, solvent carrier control, and treatment group.

3.Fluorescence spectroscopy -- Fluorescence spectroscopy is also a rapid and efficient method for estimation of population density when the only particles in suspension are algal cells. The fluorescence spectrophotometer must be calibrated according to the manufacturer's specifications before each test and calibrated to zero with growth medium that contains the highest concentration of toxicant used in the test. If toxicant fluorescence is detected at the same wavelength as algal fluorescence, this method cannot be used. The spectrophotometer should be equippped with a recorder for printed direct readout of results. The printout must be kept in the laboratory notebook.

Wavelengths of excitation and analysis must be determined (see Mitchell and Kiefer, 1984, for a discussion of absorption and excitation spectra). This is done by determining fluorescence emission at a specific emission wavelength while scanning with excitation wavelengths. The excitation wavelength at which emission was greatest is then held constant while the emission wavelength scale is scanned for greatest emission. The final excitation and emission wavelengths may be determined by a series of such steps, using a scanning range more narrow than the previous one. Wavelengths for *M. polymorphus* are approximately 443 nm (excitation) and 643 nm (emission). Examine the cultures microscopically for abnormal cellular morphology. If appreciable numbers of abnormal cells are present, estimate their percentage by differential counts of 100 cells. In order to determine the IC50 as described below, calculate the average fluorescence values for untreated control, solvent carrier control, and each treatment group. Average fluorescence of the carrier controls should be similar to average fluorescence of the untreated controls. Subtract average fluorescence of Aquil medium from average fluorescence of the control and treatment cultures for final fluorescence of the cultures.

CALCULATION OF THE IC50

The IC50 may be calculated by straight-line graphical interpolation (APHA, 1985; Walsh et al., 1987). Plot the toxicant (active ingredient in formulations) concentration on the y axis and the percentage inhibition on the x axis of semi-logarithmic graph paper. Plot the concentrations that inhibited growth just above and below 50% and draw a line between them. Interpolate the IC50 as shown in Fig. 3.

CALCULATION OF THE GROWTH RATE

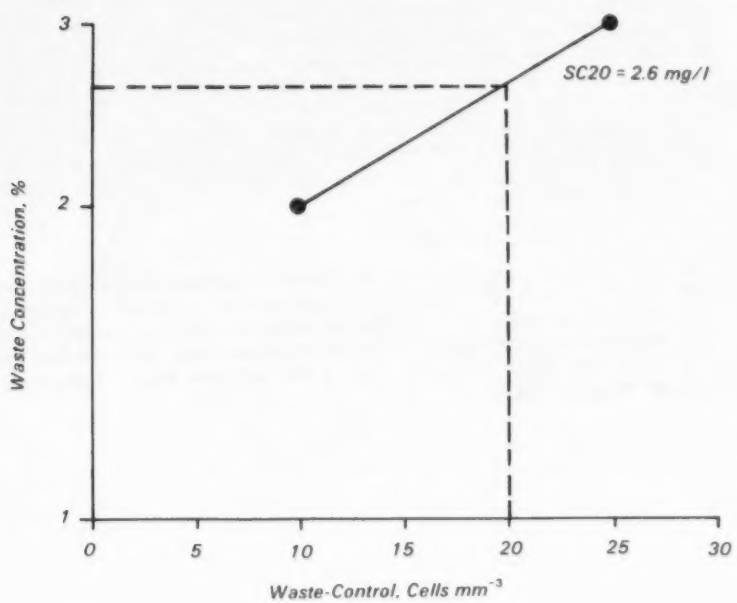
Growth rate (μ) is calculated from the expression

$$\mu = \frac{log_{10}N - log_{10}N_o}{t - t_o}$$

Where N = population density at the end of the test
N_0 = population density at the beginning of the test
$log_{10}N_0$ = 4.69
$t - t_0$ = length of time of the test (2 days).

Calculate μ from average cell numbers, optical density, or fluorescence emission of control, solvent carrier control, and each treatment group.

Figure 3. Graphical interpolation of the IC50.



2.15 Fractionation Procedure for Liquid Complex Wastes

If a complex waste inhibits or stimulates algal growth, it is often desirable to identify its bioactive constituents. This may be done by a method described by Walsh and Garnas (1983), which combines chemical fractionation and biological testing to identify bioactive organic and inorganic fractions. Bioactive fractions or subfractions may be analyzed chemically to identify toxic or stimulatory substances. Details of this procedure are given below.

2.16 Test for Living and Dead Cells

Algal growth tests do not indicate whether a toxicant simply inhibits population growth or kills cells directly. It may be desirable to know if a compound kills algae because, presumably, substances that kill are of more potential danger than those that inhibit population growth. In this case, algal populations early in the exponential growth phase are exposed to a toxicant for 24 h, and differential counts of living and dead cells are performed. Dead cells are dyed by the mortal stain, Evans blue, in contrast to unstained living cells. *Skeletonema costatum* should be used in this test because the cells are large and easy to identify.

2.17 Bioaccumulation

Algae accumulate toxicants by adsorption to the cell surface and by absorption into cells. Thus, they constitute the first link in food chain transfer, whereby toxicants may be transferred to higher trophic levels. Ability of living algae to accumulate toxicants is estimated by exposure of populations in the exponential phase of growth to various concentrations in the medium for 24 h, with subsequent analyses of cell concentrations. It is important that growing populations, where over 99% of the cells are living, are exposed because dead cells also adsorb toxicants.

2.18 Documentation and Reporting of Results

When algal tests are done for EPA a notebook that contains detailed descriptions of methods and quality control procedures used in testing must be kept in the laboratory at all times. All data from toxicity tests must be entered in this notebook and these data used for compilation of reports to EPA.

2.19 References

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3. Growth Test with Single Substances

3.1 Preparation of Toxicant

If the toxicant is soluble in growth medium at the highest concentration to be used in a test, a saturated solution in growth medium should be used as stock. Have this stock analyzed for purity and concentration.

If the toxicant is of low solubility in growth medium, several days before the test is to begin prepare a stock solution in nanograde acetone carrier on a weight to volume basis. Dissolve enough in the acetone so that, when 0.025 ml is added to 51 ml of growth medium, the concentration will approximate the saturation concentration. Have this stock analyzed for purity and concentration and store in a sealed flask in an explosion-proof refrigerator. Express results of chemical analyses as concentration of the toxic substance, whether it will be tested as a single substance or as the active ingredient in a formulation.

When toxicant purity and concentration have been confirmed, determine the highest concentration of toxicant that can be used in the test. This is done by adding 0.025 ml of the stock to 51 ml of growth medium. If crystals do not form, this concentration can be used as the highest in the toxicity test. If crystals form and do not go into solution with swirling of the medium, dilute the stock slightly and test again for crystal formation. Continue until crystals do not form. This will be the highest concentration used in the test. This method cannot be construed to estimate the saturation concentration, but it is a practical way to determine highest test concentration.

3.2 Toxicant Concentrations

The highest concentration of toxicant to be used in a test is determined as above, and lower concentrations are percentages of it. Use volumetric flasks for making dilutions of toxicant stock. In rangefinding tests, the concentrations are widely separated to estimate the IC50 and establish chemical concentrations for the definitive test. Use at least five concentrations, such as 0.01, 0.1, 1, 10, and 100% of the concentration determined above. All flasks should receive the same volume of toxicant in carrier, and acetone stock must be diluted so that 0.025 ml is added for each concentration. Convert percentage to weight of toxicant added and calculate the concentrations. Toxicant stock and dilutions should be stored in an explosion-proof refrigerator.

If the substance is toxic to algae in the rangefinding test, use at least five concentrations of toxicant that encompass the estimated IC50 in the definitive test. In most cases, each concentration should be at least 60% of the next

higher. For example, if, in the rangefinder, there was no inhibition of algal growth at 10 ppm (parts per million) and total inhibition at 100 ppm, use concentrations of 13, 22, 36, 60, and 100 ppm in the definitive test. This sequence, based on 60%, may be unacceptable in some cases, such as when there is a narrow concentration range between no inhibition and total inhibition of growth. If, for example, the IC50 is between 5 and 10 ppm, then the series, 5, 6, 7, 8, 9, and 10 ppm (six concentrations) should be used.

It is clear from the above that there cannot be a single method for determining concentrations in definitive tests, but that they must be chosen in relation to algal response. Overall, response must be graded in relation to the series of concentrations. It is best if at least one concentration inhibits growth by between 55 and 75%, and another concentration inhibits growth by between 25 and 45%. These criteria insure that responses to concentrations used to calculate the IC50 are statistically different from each other, and that population densities in treated flasks are significantly different from densities in control flasks.

3.3 Test Procedure

Day 1

a. Prepare 2 L of algal growth medium (chelexed or nonchelexed) as described on page 24; dispense 50 ml into each of 40 125-ml culture flasks; cap with stainless steel closures or previously unused foam plugs; label the flasks:
   3 control flasks
   3 solvent carrier flasks
   15 treatment flasks labeled with toxicant concentrations (3 flasks for each concentration)
   5 flasks for chemical analyses
   1 flask for inoculum culture
   1 flask for determination of pH
   1 flask to serve as a blank when population density is to be measured by light or fluorescence spectroscopy
   5 flasks that may be needed to replace flasks in which salts have precipitated
   1 flask for dilution of algal inoculum culture
   5 extra flasks to be used if needed;
   sterilize by heating in an oven at 60 C for 4 h.

b. The following method is recommended for preparation of the algal growth medium:
   1. Dissolve all salts, except $MgCl_2 \cdot 6H_2O$ in 1.5 L of glass-distilled or deionized water in a 2-L volumetric flask.
   2. After dissolution of salts, add the $MgCl_2 \cdot 6H_2O$
   3. After dissolution of $MgCl_2 \cdot 6H_2O$, add 0.5 ml of the PO_4^-, NO_3^-, and SiO_3^- nutrients; take to volume and determine salinity; add 50 ml to culture flasks as above; sterilize.

c. Prepare stock toxicant solution and its dilutions in volumetric flasks according to the instruction on page 31; wrap masking tape around the stoppers and flask necks to reduce loss by evaporation; store in the dark at 4 C in an explosion-proof refrigerator.

Day 2

a. Examine each flask for presence of precipitated salts by swirling; if precipitate is present, replace the flask with one prepared for this purpose.
b. Mix 7.5 ml of metal solution with 7.5 ml of vitamin solution; filter sterilize as described on page 23; add 0.25 ml of the metals/vitamins mix to each flask with an Eppendorf pipette fitted with a sterile tip; flame the mouth of the flask before and after each addition
c. Prepare an algal inoculum culture by sterile addition of 4-5 ml of algae from a culture in the logarithmic phase of growth to the flask prepared on Day 1; place this culture on a shaker at 60 excursions min^{-1} in the culture chamber dedicated only to algal tests; incubate for 3 days under conditions identical to those of the test.

Day 5

a. Measure pH of the medium in the flask prepared on Day 1; if it is 7.9 - 8.3, continue the test; if not, prepare new medium.
b. The inoculum culture should be in the early exponential growth phase. Immediately before use, dilute to 500 cells mm^{-3} by addition of growth medium prepared for dilution. This is done by pouring dilution medium into the inoculum culture by sterile means and estimating population density visually. A sample is taken with a sterile pipette, added to a hemocytometer, and cells over the four corner squares and one central square are counted on both sides of the hemocytometer. At this point, cell number should be greater than 500 cells mm^{-3}. By careful addition of dilution medium, cell number can be adjusted downward to a total of approximately 500 cells mm^{-3} on both sides of the hemocytometer.
c. Add 1 ml of diluted algal inoculum culture to all control, carrier control, and test flasks with an Eppendorf pipette fitted with a sterile tip; do not add algae to flasks prepared for chemical analysis; flame the mouth of each flask before and after each addition; swirl the inoculum culture between each addition to keep the cells in suspension.
d. Do not add anything more to the three untreated control flasks.
e. With no flame present, add 0.025 ml of carrier to media in the three carrier control flasks; add 0.025 ml of carrier to medium in one flask to serve as control when a spectrophotometric method is used to estimate population density; swirl each flask gently after addition of carrier; do not flame the mouths of the flasks when adding carrier.
f. Add each concentration of diluted stock toxicant to the proper set of three test flasks and to each flask prepared for chemical analysis; swirl each flask gently after addition of toxicant; do not flame the mouths of the flask when adding toxicant in carrier.
g. Place the flasks, except those prepared for chemical analysis, on shakers at 60 excursions min^{-1} in the culture chamber dedicated only to algal tests.
h. Measure light intensity at the level of the flasks.

i. Analyze for the toxicant in the media prepared for chemical analysis to confirm exposure concentrations.

Day 6

a. Measure light intensity at the level of the flasks.
b. Remove all flasks from the shakers and replace at random to insure equal illumination of cultures.

Day 7

a. Measure light intensity at the level of the flasks.
b. Remove flasks from the culture chamber; estimate population density by cell counts or absorbance or fluorescence spectroscopy by methods given in Chapter 2 of this manual.
c. Examine all cultures for presence of abnormal cells; if they are present, estimate their numbers by the method given on page 26.
d. In rangefinding tests, estimate the IC50 and conduct a definitive test with five concentrations of toxicant that encompass the estimated IC50.
e. In definitive tests, calculate the IC50 according to instructions on page 27; calculate the growth rates of control, carrier control, and test groups as shown on page 28.
f. Report results on the Reporting Form For Single Substance Analysis.

Reporting Form for Single Substance Analysis

Testing Laboratory	EPA Contract No.
Person in charge	Phone No.
Name of Chemical Compound	CAS No. Manufacturer
Generic	Date Received
Trademark	Source
Stated Purity (%)	
Other Substances in formulation and percentage active ingredient.	

Toxicant Stock Solution

Date of preparation	Solvent
Prepared by:	
Method of Chemical analysis	
Nominal Concentration	Analyzed Concentration
Purity (%)	Analyzed Purity (%)

Test conditions

Date Begin Date End Algal Species

pH___ Salinity___ Light Intensity:
High___Low___

Temperature:
High_____Low_____ Enclose a copy of the Temperature Record

Data recorded in Laboratory Notebook Number_____

Test Results

Estimation of Population Density by (Circle):
Cell Counts Optical Density Fluorescence

Range Finder Test		Definitive Test	
Toxicant Concentration	Average density	Toxicant Concentration	Average density
1. Control		1. Control	
2. Carrier Control		2. Carrier Control	
3.		3.	
4.		4.	
5.		5.	
6.		6.	
7.		7.	
8.		8.	

IC50_____ LC50_____ SC20_____

Growth Rate (μ) Toxicant Concentration μ	Cells of Abnormal Morphology (%) Toxicant Concentration %
1. Control	1. Control
2. Carrier Control	2. Carrier Control
3.	3.
4.	4.
5.	5.
6.	6.
7.	7.
8.	8.

4. Growth Tests with Liquid Complex Wastes

Initial growth tests with liquid complex wastes are conducted on unmodified whole waste. If the waste inhibits and/or stimulates algal growth, it may be desirable to fractionate it to determine if effects are caused by organic or inorganic constituents. The method outlined below was described by Walsh and Garnas (1983). It describes analysis of whole waste and fractionation procedures for use with algal tests (Figure 4).

4.1 Collection of Waste

Samples of liquid waste should be collected, shipped, and stored in glass vessels with Teflon lids or in 20-L polyethylene containers, such as the Cubitainer® (Cole Parmer Industrial Co., Chicago, IL) or its equivalent. Label the waste clearly with the name of the site, date, and other data pertinent to the collection. Give the sample a code number to be used for identification at all steps in the testing procedure. The waste should be transported to the laboratory immediately after the sample is taken and, when possible, analysis should be made immediately upon receipt. Do not store the waste for more than three days before the first test is done. Samples should be kept at approximately 4 C during shipment and storage. When received, note color, odor, and presence of solids, and measure salinity and pH.

4.2 Filtering of Waste

Waste should not be filtered, even though particles may be present, except when it contains algae. When received, examine it at approximately 100 X magnification. If algae are present, filter the waste through a 0.45 µm glass-fiber filter at no more than 0.5 atm pressure vacuum. Pass 1 L of glass-distilled or deionized water through the filter before filtering the waste.

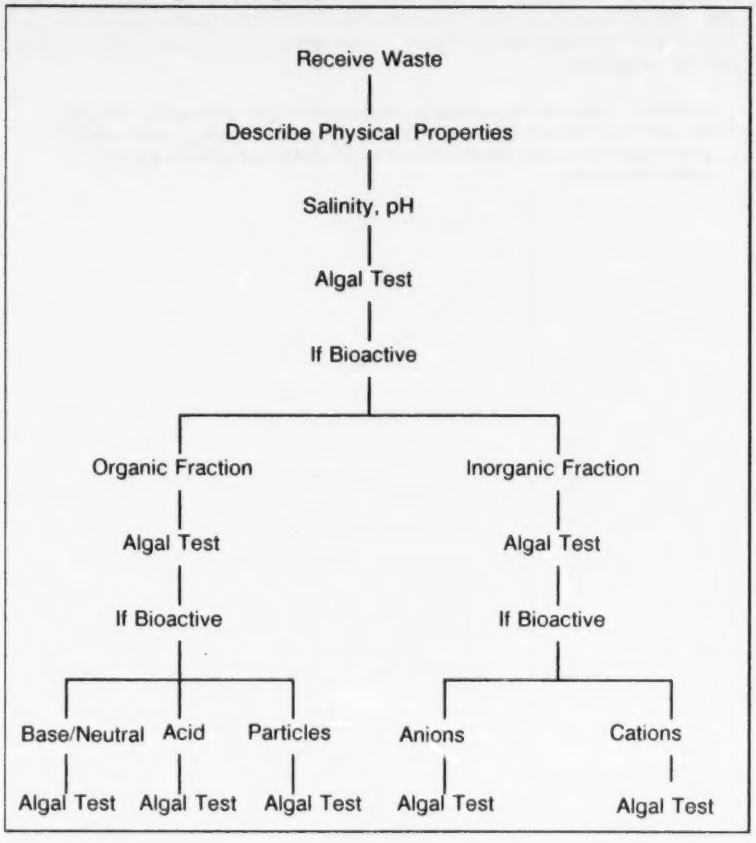
4.3 Sterile Techniques

Although the waste is not sterilized, sterile techniques should be used in all steps of the procedure to avoid addition of bacteria not found at the collection site.

4.4 Estimation of Population Density

Many liquid complex wastes contain particulates and color that preclude estimation of population density by electronic particle counters and by light or fluorescence spectroscopy. If spectroscopic methods are used, blank media prepared from each waste concentration must be used to calibrate the

Figure 4. Scheme for detecting bioactivity of complex waste and its fractions with algal toxicity tests.



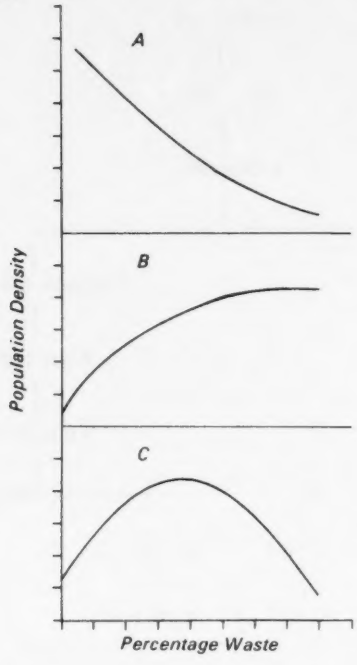
instrument to zero before cultures exposed to each concentration are analyzed. If the waste is highly colored, absorbance spectroscopy may not be used. If the waste medium fluoresces at the same emission wavelength as the algae, fluorescence spectroscopy may not be used.

In most cases, population density must be estimated by cell counts using a hemacytometer.

Examine the cultures microscopically for abnormal cellular morphology. If appreciable numbers of abnormal cells are present, estimate their percentage by differential counts of 100 cells. Do this for all tests on waste.

Calculate the average cell count, optical density, or fluorescence emission of untreated control and all waste cultures. Waste may also stimulate growth, or it may stimulate growth at low concentrations and inhibit it at higher concentrations. Figure 5 illustrates some types of responses that can be expected.

Figure 5. Types of responses of unicellular algae to complex liquid wastes: A inhibition of growth; B stimulation of growth; C stimulation of growth at low concentrations and in inhibition of growth at high concentrations.



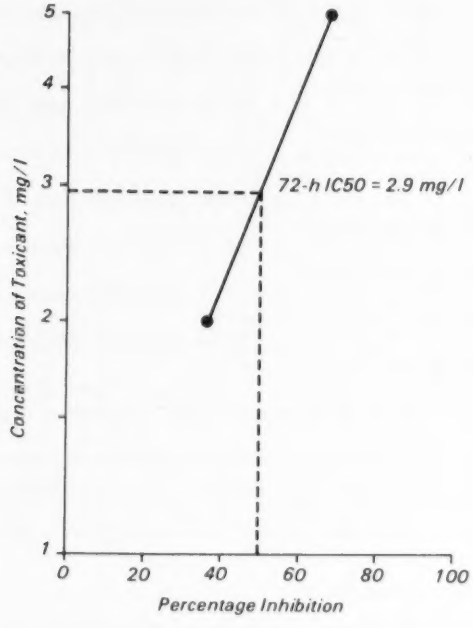
4.5 Calculation of Effect Concentration

The IC50 is calculated by straight-line graphical interpolation as for tests with single substances. A close approximation of the SC20 can be derived by the same method if average cell numbers, optical density, or fluorescence emission of cultures grown in medium made from waste are greater than 20% above those of the controls. Calculate 120% of the control value and subtract the averages of cultures just above and below that value. For example, the data should be plotted as in Fig. 6, with the interpolated SC20 value of 2.6%.

Calculate the growth rate, μ, for control, solvent carrier control, and all populations exposed to waste as described for tests with single substances.

	% Waste	Waste - Control
Control	100	-
1	102	-
2	105	-
3	110	10
4	125	25
5	160	-

Figure 6. Graphical interpolation of the SC20.



4.6 Procedure for Effects of Whole Waste

4.6.1 Preparation of Medium

The first test should be done on whole waste, filtered only if algae are present, without sterilization. After stirring to achieve homogeneity, remove 2 L and add the basic salts (prepared earlier), PO_4^-, NO_3^-, and SiO_3^-. Add the metals and vitamin mixture as for the test with single compounds, but use a metal working mix without EDTA.

Store unused waste in the dark at 4 C because it may be needed for further tests or for fractionation. Because waste may change during storage, subsequent tests should be conducted as soon as possible.

It is best to attempt a definitive test on complex waste immediately in order to avoid possible effects of storage on toxicity or growth stimulation. Dilute the waste that contains salts, nutrients, metals (without EDTA), and vitamins with similar growth medium (without EDTA) prepared with glass-distilled or deionized water. Make dilutions of 0.001, 0.01, 0.1, 1, 5, 10, 25, 50, and 75% as follows:

1. 2 L of waste = 100%
2. Add 750 ml of 1. to a 1-L volumetric flask and take to volume with growth medium = 75%
3. Add 500 ml of 1. to a 1-L volumetric flask and take to volume with growth medium = 50%
4. Add 125 ml of 3. to a 250-ml volumetric flask and take to volume with growth medium = 25%
5. Add 100 ml of 4. to a 250-ml volumetric flask and take to volume with growth medium = 10%
6. Add 125 ml of 5. to a 250-ml volumetric flask and take to volume with growth medium = 5%
7. Add 50 ml of 6. to a 250-ml volumetric flask and take to volume with growth medium = 1%
8. Add 25 ml of 7. to a 250-ml volumetric flask and take to volume with growth medium = 0.1%
9. Add 25 ml of 8. to a 250-ml volumetric flask and take to volume with growth medium = 0.01%
10. Add 25 ml of 9. to a 250-ml volumetric flask and take to volume with growth medium = 0.001%

These dilutions were designed to be made in flasks of standard volume with a minimum amount of diluted waste left after addition to test flasks.

Prepare three test flasks for each waste concentration by dispensing 50 ml into each of three 125 ml Erlenmeyer flasks. Also prepare three flasks with growth medium (without EDTA) as controls and an extra flask of each concentration to serve as blanks if spectrophotometric methods will be used for estimation of population density.

4.6.2 Procedural Steps

Day 1

a. Prepare 3 L of algal growth medium (page 21) in a 3-L Erlenmeyer flask; deliver 100 ml into each of two 250 ml

Erlenmeyer flasks and 50 ml into each of four 125 ml test flasks; place a cotton plug in the mouth of each Erlenmeyer flask and cap the mouths of the test flasks with stainless steel closures; sterilize all flasks with medium by heating at 60 C for 4 h; store the sterile medium in a draft- and dust-free cabinet.

b. Sterilize 40 125 ml culture flasks capped with stainless steel closures, a 2-L volumetric flask, and a 50-ml dispensing pipette by heating at 60 C for 4 h.

Day 2

a. Measure pH of the medium in one of the 50-ml samples prepared on Day 1; if the pH is between 7.9 and 8.3 continue with the tests; if not, prepare new medium.

b. Add 0.25 ml of filter-sterilized (page 23) metal/vitamin mix to the flask with 100 ml medium with an Eppendorf pipette fitted with a sterile tip; begin an algal inoculum culture in one of them by adding 2 - 4 ml of an algal culture in the logarithmic phase of growth with an Eppendorf pipette fitted with a sterile tip; add 0.25 ml of the mixture of metals (without EDTA) and vitamins to each flask with 50 ml of medium prepared yesterday with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.

Note: If algae are present and the waste filtered, absorbance or fluorescence methods may be used to estimate population density. See page 39 for restrictions on use of spectroscopic methods. When such methods are used, it is necessary to prepare an extra flask with each dilution and undiluted waste to serve as blanks.

Day 5

a. With the waste at room temperature, add salts and nutrients (Table 1) to 2 L of waste in the sterile volumetric flask. Dissolve all salts first except $MgCl_2 \cdot 6H_2O$, then the $MgCl_2 \cdot 6H_2O$; add 0.5 ml of the PO_4^-, NO_3^-, and SiO_3^- nutrients; then add 10 ml of filter-sterilized metals/vitamin mix (the metals should not contain EDTA); take to volume with the remaining waste.

b. Dilute the waste to which salts and nutrients were added with medium prepared on Day 1 according to the method given on page 42.

c. Dispense 50 ml of undiluted waste and each dilution into each of three 125-ml test flasks with the sterile dispensing pipette; this will include 3 flasks for each of the 9 dilutions and 3 flasks for undiluted waste; label each flask with identifying code, dilution, and date.

d. Measure the pH of undiluted waste and all dilutions with the portions remaining after addition to the test flasks; if fluorescence will be used to estimate population density in filtered medium, determine if the waste fluoresces at excitation and analysis wavelengths to be used; if it does, fluorescence may not be used to estimate population density.

e. Prepare algal inoculum culture by diluting the culture begun on Day 2 with a portion of the remaining 100 ml of medium

prepared on Day 1; dilute to 500 cells mm^{-3} by the method on page 33.

f. Use 3 of the 125-ml culture flasks that contain 50 ml of medium as untreated controls.

g. Add 1 ml of the diluted algal inoculum culture to each flask with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.

h. Place the flasks at random on shakers at 60 excursions min^{-1} in the culture chamber dedicated to algal tests.

i. Measure intensity of light at the level of the cultures.

Day 6

a. Measure intensity of light at the level of the cultures.

b. Remove all flasks from the shakers and replace at random.

Day 7

a. Measure intensity of light at the level of the cultures.

b. Remove the algal cultures from the culture chamber and estimate population density of each by cell counts or by absorbance or fluorescence spectroscopy; if the waste is not filtered, count the number of cells as described on page 26; if the waste is filtered, population density may be estimated by cell counts or by absorbance or fluorescence spectroscopy.

c. If population density is estimated by absorbance, use each dilution and the undiluted waste (see note on page 43) to zero the instrument before measuring absorbance of cultures at comparable dilutions and undiluted waste, or subtract their absorbances from absorbances of comparable test cultures.

d. Calculate the IC50, SC20, or both, as described on page 27 and 40; calculate the average growth rate for control and all waste concentration groups as described on page 28.

e. Report results on the Reporting Form For Complex Waste Analysis.

4.7 Procedures for Effects of Inorganic and Organic Fractions

If whole waste is biologically active, and it is desirable to categorize broadly its toxic or stimulating components, it may be fractionated into organic and inorganic components and subfractionated into anion and cation inorganics and acid and base/neutral extractable organics. Also, substances extracted from particulate matter that is part of the waste may be tested.

Tests on the fractions and subfractions obtained by the methods described here identify bioactive portions of the waste. They cannot be construed to express toxicity with great accuracy because of problems associated with the extraction procedures and because of possible effects of solvents on algal population growth. Further tests may be required to identify individual toxic components.

4.7.1 Preparation and Testing of Inorganic and Organic Fractions

On the day that the test on whole waste is placed in the algal growth chamber, mix the waste well, place 2 L in a glass container, and store in the dark at approximately 4 C. Do not freeze the waste. This portion is to be used

for a test on the whole waste if the first test must be repeated. The remaining 16 L of whole waste will be used for fractionation.

Materials

1. 142 mm filter holder
2. 142 mm glass-fiber filter
3. Silicon tubing 1/8" id x 1/4" od
4. 20 L Cubitainer
5. Microflow adjustable gear pump
6. Disposable syringe with Luer Lock® (Becton, Dickenson & Co., Rutherford, NJ) tip, 50 ml
7. Amberlite XAD-4 resin
8. Dowex® (Dow Co., Midland, MI) 1-X8 strong base anion exchange resin
9. Dowex 50W-X8 strong acid cation exchange resin, 20 - 50 mesh
10. 20 mm nylon mesh screen
11. 50 Kg scale

XAD-4 Resin Preparation

1. Rinse the resin repeatedly in an Erlenmeyer flask with glass-distilled or deionized water until all salts and fines are removed. Allow the remaining resin beads to stand in 2N H_2SO_4 for 30 min and then rinse with glass-distilled or deionized water.
2. Remove impurities by swirling the resin with technical grade acetone intermittently for 30 min, decant, and remove the remaining organic contaminants by sequential solvent extractions with acetone and methanol in a Soxhlet extractor for 12 h for each solvent.
3. Transfer the purified resin with distilled methanol into an Erlenmeyer flask fitted with a ground glass stopper for storage.

Column

The column consists of a 50-ml disposable syringe loosely plugged with glass wool and filled with 50 ml (wet volume) XAD-4 resin. Use at least 20 bed volumes of glass-distilled or deionized water to displace methanol from the column. Connect a bored No. 6 Neoprene® stopper coupled to a 3-cm piece of 8 mm od glass tubing to the top of the column. A number of columns can be prepared this way and stored in a refrigerator until needed.

Filter

Position a glass-fiber filter in the filter holder and overlay with 20 µm nylon mesh to prevent the filter from clogging.

Quality Assurance

In conjunction with the large-volume extraction method, 1-L aliquots of water sampled before and after resin treatment should be extracted according to procedures in the "Method 625 Base/Neutrals and Acids" (Office of the Federal Register, 1986). The fractions obtained from this procedure, i.e., organic base/neutral and acid can be tested for effects on algal growth.

Fractionation System

1. Following initial testing of raw effluent, weigh the Cubitainer and its contents to 0.1 Kg, add a 7.5-cm Teflon stirring bar to the container, and connect a service faucet to the opening.

2. Connect the Cubitainer to the inlet of the filter assembly with a minimal length of silicon tubing and connect the column to the filter exit and to the inlet of the microflow pump with silicon tubing. Direct the exit of the pump into an empty, tared Cubitainer to receive the filtered, extracted sample. Determine volume of the sample gravimetrically and store in the dark at approximately 4 C.
3. Be sure to stir the raw effluent in the Cubitainer continuously to insure homogeneous distribution of solids, and adjust the pump so that the flow through the apparatus is 500 to 1000 ml h^{-1}. Care must be taken to avoid overload of the column with subsequent breakthrough of organics.
4. Following extraction of organics, remove the Neoprene stopper and aspirate the resin by vacuum to remove excess water. Fit the disposable syringe with an 18 gauge stainless steel needle and elute with 150 ml nanograde acetone into a rotary evaporator concentrator flask. Concentrate this organic fraction by vacuum to 25 ml at room temperature and store, tightly capped, in an explosion-proof refrigerator.

4.7.2 Procedural Steps

Day 1 — Prepare XAD-4 resin and make a resin column as described on page 45.

Day 2
a. Prepare 3 L of algal growth medium in a 3-L flask (page 24), adding 7.5 ml of each of the PO_4^-, NO_3^-, and SiO_3^- solutions; dispense 50 ml into each of four 125-ml culture flasks and cap with stainless steel closures; dispense 100 ml into each of two 250-ml Erlenmeyer flasks and plug with cotton stoppers; plug the 3-L flask with a cotton stopper; sterilize all flasks with medium by heating at 60 C for 4 h; store in a draft- and dust-free cabinet.
b. Sterilize 40 125-ml culture flasks capped with stainless steel closures, two 2-L volumetric flasks, and two 50-ml dispensing pipettes by heating at 60 C for 4 h.
c. Pass the remaining 16 L of waste through the XAD-4 resin column as described on page 45. Column effluent will contain the inorganic fraction and, perhaps, ionized organics. This fraction will be called the inorganic fraction, even though, in some instances, it may contain a small amount of organic material. Store this inorganic fraction in the dark at approximately 4 C. The organic fraction is the material that remains on the XAD-4 resin column.
d. Extract the organic fraction from the resin with acetone and reduce the volume to 25 ml as described above; store in an explosion-proof refrigerator.

Day 3
a. Measure pH of the medium in one of the 50-ml samples prepared yesterday; if the pH is between 7.9 and 8.3 continue with the test; if not, prepare new medium.
b. Add 0.5 ml of filter-sterilized (page 23) metal/vitamin mix to the flasks with 100 ml medium with an Eppendorf pipette fitted

with a sterile tip; begin an algal inoculum culture in one of them by adding 4-5 ml of an algal culture in the logarithmic phase of growth with an Eppendorf pipette fitted with a sterile tip; add 0.25 ml of the mix of metals (without EDTA) and vitamins to each of the flasks with 50 ml of medium prepared yesterday with an Eppendorf pipette fitted with a sterile tip, flame the mouth of each flask before and after each addition.

Preparation and Testing of the Inorganic Fraction

Day 6

a. Prepare algal growth medium with the inorganic column effluent 6 with salts as described on page 24; add the salts to approximately 1.5 L of column effluent with continuous stirring in the sterile 2-L volumetric flask; when the salts have dissolved, add 0.5 ml of each of the PO_4^-, NO_3^-, and SiO_3^- nutrients and 10 ml of the mixture of metals (without EDTA) and vitamins with an Eppendorf pipette fitted with a sterile tip; take to volume with the inorganic column effluent and mix thoroughly; store the remaining column effluent in the dark at approximately 4 C; if fluorescence spectroscopy is to be used to estimate algal population density, determine if this sample fluoresces at the excitation and analysis wavelengths required for the test algal species; if it does, fluorescence cannot be used; if absorbance will be used to estimate population density, determine if color present in the fraction is detectable at the wavelength used for the algal species; if it is, prepare an extra flask, as described in c below, for undiluted medium and all dilutions; use these as blanks for the zero spectrophotometer settings for comparable test cultures, or subtract their absorbance values from those of comparable test cultures.

b. Add 5 ml of sterile mix of metals (without EDTA) and vitamins to each liter of dilution medium prepared on Day 2; dilute the medium prepared from inorganic column effluent according to instructions on page 42.

c. Dispense 50 ml of undiluted medium and each dilution into 3 sterile test flasks prepared on Day 2 with the sterile dispensing pipette; this will include 3 flasks for each of the 9 dilutions and 3 flasks for undiluted medium; label each flask with identifying code, dilution, and date.

d. Measure the pH of undiluted medium prepared from inorganic column effluent and all dilutions with the portions remaining after addition to the test flask.

e. Prepare an algal inoculum culture by diluting the culture begun on Day 3 with a portion of the remaining 100 ml of medium prepared on Day 2. Dilute to 500 cells mm^{-3} by the method on page 33.

f. Use the three flasks with 50 ml medium prepared on Day 2 as untreated controls.

g. Add 1 ml of the diluted algal inoculum culture to each flask with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.

h. Place the flasks at random on shakers at 60 excursions min^{-1} in the culture chamber dedicated to algal tests.
i. Measure intensity of light at the level of the cultures.

Day 7
a. Measure intensity of light at the level of the cultures.
b. Remove all flasks from the shakers and replace at random.

Day 8
a. Measure intensity of light at the level of the cultures.
b. Remove the algal cultures from the culture chamber and estimate population density of each by cell counts or by absorbance or fluorescence spectroscopy.
c. If population density is estimated by absorbance, use each dilution and undiluted medium (see note on page 43) to zero the instrument before measuring absorbance of cultures at comparable dilutions and undiluted medium, or subtract their absorbances from absorbances of comparable test cultures.
d. Calculate the IC50, SC20, or both, as described on pages 27 and 40; calculate the average growth rate for control and all waste concentration groups as described on page 28.
e. Report results on the Reporting Form For Chemical Fraction Analysis.

Preparation and Testing of the Organic Fraction

Day 2
a. Prepare 5 L of algal growth medium in a 6-L flask as described on page 24; add 1.25 ml of each of the PO_4^-, NO_3^-, and SiO_3^- solutions; dispense 50 ml into each of four 125-ml culture flasks and cap with stainless steel closures; dispense 100 ml into each of two 250-ml Erlenmeyer flasks and plug with cotton stoppers; plug the 3-L flask with a cotton stopper; sterilize all flasks with medium by heating at 60 C for 4 h; store in a draft- and dust-free cabinet.
b. Sterilize 40 125-ml culture flasks capped with stainless steel closures, two 2-L volumetric flasks, and a 50-ml dispensing pipette by heating at 60 C for 4 h.

Day 3
a. Measure pH of the medium in one of the 50-ml samples prepared yesterday; if the pH is between 7.9 and 8.3 continue with the test; if not, prepare new medium.
b. Add 0.5 ml of filter-sterilized metal/vitamin mix (page 23) to the flasks with 100 ml of medium with an Eppendorf pipette fitted with a sterile tip; begin an algal inoculum culture in one of them by adding 4-5 ml of an algal culture in the logarithmic phase of growth with an Eppendorf pipette fitted with a sterile tip; add 0.25 of the mixture of metals (without EDTA) and vitamins to each flask with 50-ml medium prepared on Day 2 with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.

Day 6
a. Carefully add 3.1 ml of the acetone solution of organic compounds from the XAD-4 resin column to approximately 1.5 L of growth medium prepared on Day 1 in a 2-L volumetric flask; add 5 ml of the mixture of metals (without EDTA) and vitamins; bring to volume with growth medium and mix thoroughly.

b. Dilute the medium that contains the organic extract as described on page 42.
c. Dispense 50 ml of undiluted medium and each dilution to each of three 125-ml volumetric flasks sterilized on Day 1 with the sterile dispensing pipette; this will include 3 flasks for each of 9 dilutions and 3 flasks for undiluted medium; label each flask with identifying code, dilution, and date.
d. Measure the pH of undiluted waste and all dilutions with the portions remaining after addition to the test flasks; if fluorescence is used to estimate population density in filtered medium, determine if the waste fluoresces at excitation and analysis wavelengths to be used; if it does, fluorescence may not be used to estimate population density.
e. Prepare an algal inoculum culture by diluting the culture begun on Day 2 with a portion of the remaining 100 ml of medium prepared on Day 1; dilute to 500 cells mm^{-3} by the method on page 33.
f. Use 3 of the 125-ml culture flasks that contain 50 ml of medium as untreated controls.
g. Add 1 ml of the diluted algal inoculum culture to each flask with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.
h. Place the flasks at random on shakers at 60 excursions min^{-1} in the culture chamber dedicated to algal tests.
i. Measure intensity of light at the level of the cultures.

Day 7
a. Measure intensity of light at the level of the cultures.
b. Remove all flasks from the shakers and replace at random.

Day 8
a. Measure intensity of light at the level of the cultures.
b. Remove the algal cultures from the culture chamber and estimate population density of each by cell counts or by absorbance or fluorescence spectroscopy.
c. If population density is estimated by absorbance, use each dilution and the undiluted waste (see note on page 43) to zero the instrument before measuring absorbance of cultures at comparable dilutions and undiluted waste, or subtract their absorbances from absorbances of comparable test cultures.
d. Calculate the IC50, SC20, or both, as described on pages 27 and 40; calculate the average growth rate for control and all fraction concentration groups as described on page 28.

4.8 Procedures for Effects of Inorganic and Organic Subfractions

4.8.1 Inorganic Subfractions

If the inorganic fraction of waste inhibited or stimulated algal growth, it may be desirable to know whether the effect was due to cations or anions. The following method may be used for this purpose.

4.8.1.1 Preparation and Testing of the Cation Subfraction

Day 1

a. Prepare 5 L of algal growth medium (page 24) in a 6-L Erlenmeyer flask; deliver 100 ml into each of two 250-ml Erlenmeyer flasks and 50 ml into each of four 125-ml test flasks; place a cotton plug in the mouth of each Erlenmeyer flask and cap the mouths of the test flasks with stainless steel closures; sterilize all flasks with medium by heating at 60 C for 4 h; store the sterile medium in a draft- and dust-free cabinet.

b. Sterilize 40 125-ml culture flasks capped with stainless steel closures, five 2-L volumetric flasks, and five 50-ml dispensing pipettes by heating at 60 C for 4 h.

Day 2

a. Measure pH of the medium in one of the 50-ml samples prepared yesterday; if the pH is between 7.9 and 8.3 continue with the test; if not, prepare new medium.

b. Add 0.5 ml of filter-sterilized (page 23) metal/vitamin mix to the flasks with 100 ml medium with an Eppendorf pipette fitted with a sterile tip; begin an algal inoculum culture in one of them by adding 4-5 ml of an algal culture in the logarithmic phase of growth with an Eppendorf pipette fitted with a sterile tip; add 0.25 ml of the mixture of metals (without EDTA) and vitamins to each flask with 50 ml of medium prepared yesterday with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.

Day 3-4

a. Prepare the cation subfraction by batch extraction: slowly add 2 N HCl to 2 L of the inorganic fraction until the pH is less than 4; add 20 g of Dowex 1-X8 strong base anion exchange resin; stir for 24 h; filter the resin from the sample with the glass fiber filter assembly; adjust to pH 7 with 2 N NaOH.

b. Prepare algal growth medium with the cation subfraction: do this by adding 1.5 L to a 2-L volumetric flask, dissolving first all salts except $MgCl_2.6H_2O$, then the $MgCl_2.6H_2O$, and then adding 0.5 ml of the PO_4^-, NO_3^-, and SiO_3^- nutrients; then add 10 ml of filter-sterilized metal/vitamin mix (the metals should not contain EDTA); take to volume with the remaining waste.

c. Dilute the cation subfraction to which salts and nutrients were added with medium prepared on Day 1 according to the method given on page 42.

d. Dispense 50 ml of undiluted cation medium and each dilution into each of three 125-ml test flasks with the sterile dispensing pipette, this will include 3 flasks each for the 9 dilutions and 3 flasks for undiluted waste; label each flask with identifying code, dilution, and date.

e. Measure the pH of undiluted cation medium and all dilutions with the portions remaining after addition to the test flasks; if fluorescence will be used to estimate population density in filtered medium, determine if the waste fluoresces at the

excitation and analysis wavelengths to be used; if it does, fluorescence may not be used to estimate population density.

Day 5
a. Prepare an algal inoculum culture by diluting the culture begun on Day 2 with a portion of the remaining 100 ml of medium prepared on Day 2; dilute to 500 cells mm^{-3} by the method on page 33.
b. Use 3 of the 125-ml culture flasks that contain 50 ml of medium as untreated controls.
c. Add 1 ml of the diluted algal inoculum culture to each flask with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.
d. Place the flasks at random on shakers at 60 excursions min^{-1} in the culture chamber dedicated to algal tests.
e. Measure intensity of light at the level of the cultures.

Day 6
a. Measure intensity of light at the level of the cultures.
b. Remove all flasks from the shakers and replace at random.

Day 7
a. Measure intensity of light at the level of the cultures.
b. Remove the algal cultures from the culture chamber and estimate population density of each by cell counts or by absorbance or fluorescence spectroscopy.
c. If population density is estimated by absorbance, use each dilution and the undiluted waste (see note on page.43) to zero the instrument before measuring absorbance of cultures at comparable dilutions and undiluted waste, or subtract their absorbances from absorbances of comparable test cultures.
d. Calculate the IC50, SC20, or both, as described on pages 27 and 40; calculate the average growth rate for control and all cation subfraction concentrations as described on page 28.
e. Report results on the Reporting Form For Chemical Fraction Analysis.

4.8.1.2 Preparation and Testing of the Anion Subfraction

Day 1
a. Prepare 5 L of algal growth medium (page 24) in a 6-L Erlenmeyer flask; deliver 100 ml into each of two 250-ml Erlenmeyer flasks and 50 ml into each of four 125-ml test flasks; place a cotton plug in the mouth of each Erlenmeyer flask and cap the mouths of the test flasks with stainless steel closures; sterilize all flasks with medium by heating at 60 C for 4 h; store the sterile medium in a draft- and dust-free cabinet.
b. Sterilize 40 125-ml culture flasks capped with stainless steel closures by heating at 60 C for 4 h.

Day 2
a. Measure pH of the medium in one of the 50-ml samples prepared yesterday; if the pH is between 7.9 and 8.3 continue with the test; if not, prepare new medium.
b. Add 0.5 ml of filter-sterilized (page 23) metal/vitamin mix to the flasks with 100 ml medium with an Eppendorf pipette fitted with a sterile tip; begin an algal inoculum culture in one of them

by adding 4-5 ml of an algal culture in the logarithmic phase of growth with an Eppendorf pipette fitted with a sterile tip; add 0.25 ml of the mixture of metals (without EDTA) and vitamins to each flask with 50 ml of medium prepared yesterday with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.

Day 3-4

a. Prepare the anion subfraction by batch extraction: slowly add 2 N NaOH to 2 L of the inorganic fraction until the pH is greater than 10; add 20 g of Dowex 50W-X8 strong acid cation exchange resin (20-50 mesh); stir for 24 h; filter the resin from the sample with the glass fiber filter assembly; adjust to pH 7 with 2 N HCl.

b. Prepare algal growth medium with the anion subfraction: add 1.5 L to a sterile 2-L volumetric flask, dissolve first all salts except $MgCl_2 \cdot 6H_2O$; then dissolve $MgCl_2 \cdot 6H_2O$ and add 0.5 ml of the PO_4^-, NO_3^-, and SiO_3^- nutrients; then add 10 ml of filter-sterilized metal/vitamin mix (the metals should not contain EDTA); take to volume with the remaining waste.

c. Dilute the anion subfraction to which salts and nutrients were added with medium prepared on Day 1 according to the method given on page 42.

d. Dispense 50 ml of undiluted cation medium and each dilution into each of three 125-ml test flasks with a sterile dispensing pipette, this will include 3 flasks for each of the 9 dilutions and 3 flasks for undiluted waste; label each flask with identifying code, dilution, and date.

e. Measure the pH of undiluted waste and all dilutions with the portions remaining after addition to the test flasks; if fluorescence is used to estimate population density in filtered medium, determine if the waste fluoresces at the excitation and analysis wavelengths to be used; if it does, fluorescence may not be used to estimate population density.

Day 5

a. Prepare an algal inoculum culture by diluting the culture begun on Day 2 with a portion of the remaining 100 ml of medium prepared on Day 2; dilute to 500 cells mm^{-3} by the method on page 33.

b. Use 3 of the 50-ml volume of medium in 125-ml culture flasks as untreated controls.

c. Add 1 ml of the diluted algal inoculum culture to each flask with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.

d. Place the flasks at random on shakers at 60 excursions min^{-1} in the culture chamber dedicated to algal tests.

e. Measure intensity of light at the level of the cultures.

Day 6

a Measure intensity of light at the level of the cultures.

b. Remove all flasks from the shakers and replace at random.

Day 7

a. Measure intensity of light at the level of the cultures.

b. Remove the algal cultures from the culture chamber and estimate population density of each by cell counts or by absorbance or fluorescence spectroscopy.
c. If population density is estimated by absorbance, use each dilution and the undiluted waste (see note on page 43) to zero the instrument before measuring absorbance of cultures at comparable dilutions and undiluted waste, or subtract their absorbances from absorbances of comparable test cultures.
d. Calculate the IC50, SC20, or both, as described on pages 27 and 40; calculate the average growth rate for control and all anion subfraction concentrations as described on page 28.
e. Report results on the Reporting Form For Chemical Fraction Analysis

4.8.2 Organic Subfractions

If the organic fraction of waste inhibited or stimulated algal growth, it may be desirable to know whether the effect was due to acid or base/neutral extractable organics. The extraction method is described in "Method 625 Base/ Neutrals and Acids," published in the *Code of Federal Regulations* (Office of the Federal Register, 1986). It is best for the toxicologist who works with these subfractions to consult that publication for details of the subfractionation steps and instructions for preparation of the base/neutrals and acid subfractions of the organic fraction.

4.8.2.1 Preparation and Testing of the Subfractions

Day 1
a. For each subfraction prepare 5 L of algal growth medium (page 24) in a 6-L Erlenmeyer flask; deliver 100 ml into each of two 250-ml Erlenmeyer flasks and 50 ml into each of four 125-ml test flasks; place a cotton plug in the mouth of each Erlenmeyer flask and cap the mouth of each test flask with stainless steel closures; sterilize all flasks with medium by heating at 60 C for 4 h; store the sterile medium in a draft- and dust-free cabinet.
b. Sterilize 40 125-ml culture flasks capped with stainless steel closures by heating at 60 C for 4 h.

Day 2
a. Measure pH of the medium in one of the 50-ml samples prepared yesterday; if the pH is between 7.9 and 8.3 continue with the test; if not, prepare new medium.
b. Add 0.5 ml of filter-sterilized (page 23) metal/vitamin mix to the flasks with 100 ml medium with an Eppendorf pipette fitted with a sterile tip; begin an algal inoculum culture in one of them by adding 4-5 ml of an algal culture in the logarithmic phase of growth with an Eppendorf pipette fitted with a sterile tip; add 0.25 ml of the mixture of metals (without EDTA) and vitamins to each of the flasks with 50 ml of medium prepared yesterday with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.
c. Add 3.1 ml of the organic fraction to 2 L of distilled or deionized water and extract according to instructions given for

separatory funnel extraction in "Method 625-Base/Neutrals and Acids" of the *Code of Federal Regulations*. This will yield two organic subfractions: base/neutral and acid in methylene chloride. Before testing, exchange the methylene chloride with dimethyl sulfoxide, which is less toxic to algae. Take the volume to 2 ml and store as instructed in Method 625.

d. The following instructions (Day 6) describe testing with both extracts. Testing may be done on each at the same or different times.

Day 6

a. Add 2 ml of the base/neutrals or acids extract to approximately 1.5 L of algal growth medium prepared on Day 1 in a sterile 2-L volumetric flask; add 5 ml of the mix of metals (without EDTA) and vitamins; bring to volume with growth medium and mix thoroughly.

b. Dilute the medium that contains the organic subfraction as described on page 42.

c. Dispense 50 ml of undiluted medium and each dilution into each of three 125-ml test flasks with a sterile dispensing pipette, this will include 3 flasks for each of 9 dilutions and 3 flasks for undiluted waste; label each flask with identifying code, dilution, and date.

d. Measure the pH of undiluted medium and all dilutions with the portions remaining after addition to the test flask; if fluorescence is used to estimate population density in filtered medium, determine if the waste fluoresces at excitation and analysis wavelengths to be used; if it does, fluorescence may not be used to estimate population density.

e. Prepare an algal inoculum culture by diluting the culture begun on Day 2 with a portion of the remaining 100 ml of medium prepared on Day 2; dilute to 500 cells mm^{-3} by the method on page 33.

f. Use 3 of the 125-ml culture flasks that contain 50 ml of medium as untreated controls.

g. Add 1 ml of the diluted algal inoculum culture to each flask with an Eppendorf pipette fitted with a sterile tip; flame the mouth of each flask before and after each addition.

h. Place the flasks at random on shakers at 60 excursions min^{-1} in the culture chamber dedicated to algal tests.

i. Measure intensity of light at the level of the cultures.

Day 7

a. Measure intensity of light at the level of the cultures.

b. Remove all flasks from the shakers and replace at random.

Day 8

a. Measure intensity of light at the level of the cultures.

b. Remove the algal cultures from the culture chamber and estimate population density of each by cell counts or by absorbance or fluorescence spectroscopy.

c. If population density is estimated by absorbance, use each dilution and the undiluted waste (see note on page 43) to zero the instrument before measuring absorbance of cultures at

comparable dilutions and undiluted waste, or subtract their absorbances from absorbances of comparable test cultures.

d. Calculate the IC50, SC20, or both, as described on pages 27 and 40; calculate the average growth rate for control and all subfraction concentrations as described on page 28.
e. Report results on the Reporting Form For Chemical Fraction Analysis.

4.9 Procedures for Extraction and Testing of Particles

The following procedure may be used to determine if organic materials that can be extracted from waste particulate matter are toxic.

4.9.1 Extraction of Organics

1. Remove the glass fiber filter and nylon screen from the filter holder; cut into pieces; place in a 150-ml Corex® (Corning Glass Works, Corning, NY) bottle.
2. Extract the filter pieces 3 times with 50 ml nanograde acetone using a Branson Sonic Probe for 5 min with each extraction.
3. Centrifuge the extract at 5000 RPM.
4. Concentrate the combined acetone extracts by vacuum at room temperature to 25 ml.

4.9.2 Testing of Extract

1. The extract contains organics from the particulate matter in 16 L of waste
2. Test this extract with the method "Preparation and Testing of the Organic Fraction" page 48.

4.10 References

Office of the Federal Register. 1986. Method 625-base/neutrals and acids. pp. 442-469. Code of Federal Regulations, 40 CFR 104.1, Revised July 1, 1986, U.S. Government Printing Office, Washington, DC.

Walsh, G.E. and R.L. Garnas. 1983. Determination of bioactivity of chemical fractions of liquid wastes using freshwater and saltwater algae and crustaceans. Environ. Sci. Technol. 17: 180-182.

Complex Waste Analysis Reporting Form		
Testing Laboratory		EPA Contract No.
Person in charge		Phone No.
Origin of Waste		
Name of Site		Address
Type of Industry		
Sample Collected By		Date Collected
Method of Collection		Date Received
Method of Transport		Time of Transport
Log No.		
Color pH Salinity Odor Solids		
Other Applicable Characteristics		
Test Conditions		
Date Begin	Date End	Algal Species
Light Intensity: High___ Low___ Temperature: High_____Low_____ Enclose copy of Temperature Record Data recorded in Laboratory Notebook Number_____		

Test Results

Estimation of Population Density by (Circle):
Cell Counts Optical Density Fluorescence

First Test		Second Test (If Necessary)	
Waste Conc (%)	Average density	Waste Conc (%)	Average density
1. Control		1. Control	
2.		2.	
3.		3.	
4.		4.	
5.		5.	
6.		6.	
7.			
8.			
9.			
10.			
11.			

IC50_____ LC50_____ SC20_____

Chemical Fraction Analysis Reporting Form			
Test Conditions Date Begin Date End Algal Species			
Light Intensity: High_____ Low_____ Temperature: High_____ Low_____ Data recorded in Laboratory Notebook Number_____			
Test Results, Fractions Estimation of Population Density by (Circle): Cell Counts Optical Density Fluorescence Fraction_____			
Range Finder Test		Definitive Test	
% Original Conc.	Average Density	% Original Conc.	Average Density
1. Control		1. Control	
2.		2.	
3.		3.	
4.		4.	
5.		5.	
6.		6.	
IC50_____	LC50_____		SC20_____

Growth Rate (μ)	Cells of Abnormal Morphology
Waste Concentration (%) μ	Waste Concentration (%) %
1. Control	1. Control
2.	2.
3.	3.
4.	4.
5.	5.
6.	6.
7.	7.
8.	8.
9.	9.
10.	10.
11.	11.

5. Bioaccumulation Test

It may be important to know if algae accumulate a compound. This is determined by exposure of a population for 24 h, with subsequent chemical analysis.

1. Dispense 1 L of algal growth medium into six 2.8-L Fernbach flasks, seal with a foam plug or cotton stopper, and sterilize in an oven at 60 C for 4 h.
2. Add 100 ml of an algal stock culture in the exponential phase of growth to the flasks and incubate in a growth chamber for five days. Continuous light at 100-120 $\mu E\ m^{-2}\ sec^{-1}$ should be supplied at 20 C. Swirl the flasks twice each day to maintain gaseous exchange between atmosphere and growth medium.
3. On Day 5, add toxicant at the highest concentration that does not kill algae (see Enumeration of Dead Cells Section) to five flasks, and return the cultures to the growth chamber for 24 h.
4. After 24 h exposure, centrifuge the cultures at 5000 rpm and combine all treated algae by resuspension in fresh medium (without toxicant); add them to a previously weighed centrifuge tube and centrifuge again.
5. Weigh the tube with algae and subtract the weight of the tube to determine the weight of algae.
6. Analyze for toxicant concentration in algae from the control and treated flasks and calculate uptake as weight of toxicant per milligram of wet cells versus weight of toxicant per milliliter of growth medium. Calculate the concentration factor:

$$\text{Concentration Factor} = \frac{\text{toxicant concentration in algae}}{\text{toxicant concentration in water}}$$

7. If concentration of toxicant per milligram of dry algae is desired:
   a. Dry a filter to constant weight at 110 C.
   b. Collect a known wet weight of algal cells suspended in growth medium on the filter by very gentle vacuum.
   c. Dry again to constant weight at 110 C and weigh to the nearest 0.1 mg.
   d. Subtract the weight of the filter from the weight of the filter with algae for dry algal weight.
   e. Calculate the concentration factor as described above.
   f. Report results on the Bioaccumulation Studies Reporting Form.

NOTE:This method cannot be used if the toxicant is volatile at 110 C. There is a small error in estimation of dry weight of algae by the above method.

Some of the salt from the growth medium will be present on the cells and filter after drying. This is small in relation to algal weight and may be ignored in this test.

Test Results			
Toxicant Concentration in Test Nominal		Analyzed	
Bioconcentration	Toxicant Concentration Algae	Toxicant Concentration Medium	Conc. in Algae/ Conc. in Medium
1. Control			
2.Treated			

Bioaccumulation Studies Reporting Form	
Testing Laboratory	EPA Contract Number
Person in charge	Phone Number
Name of Compound	CAS No.
Chemical	Manufacturer
Generic	Date Received
Trademark	Source
Stated Purity (%)	
Other Substances in Formulation and Percentage Active Ingredient	
Toxicant Stock Solution	
Date of Preparation Solvent	
Prepared by	
Method of Chemical Analysis	
Nominal Concentration	Analyzed Concentration
Purity (%)	Analyzed

Test Conditions

Date Begin_____ Date End_____

Algal Species pH_____ Salinity_____

Light Intensity: High_____ Low_____

Temperature:
High_____ Low_____ Enclose copy of Temperature Record

Data recorded in Laboratory Notebook Number_____

6. Enumeration of Living and Dead Cells

The method used here for detection and enumeration of dead algal cells was described by Crippen and Perrier (1974); Reynolds et al. (1978) and Walsh (1983) used it to detect effects of toxicants on marine unicellular algae. Alexander and Wilkinson (1987) showed that toxicants may affect the pattern of staining.

1. Prepare a 1% (w/v) solution of the acidic mono-azo dye, Evans blue, by dissolving 1 g of the dye in 100 ml of algal growth medium.
2. Prepare an algal test with *Skeletonema costatum* as described for single substances but do not add toxicant. After 48 h, remove the algal cultures from the growth chamber and add toxicant and solvent carrier as for the test with single substances. Return the cultures to the growth chamber and incubate for 24 h.
3. After 24-h exposure, centrifuge the culture gently and resuspend the cells in a selected volume of uncontaminated medium. Centrifugation should not disrupt cells; time and speed of centrifugation must be determined by trial and error. Dispense 10 ml of the suspension into a test tube.
4. Add 0.5 ml of the Evans blue solution to the 10 ml of algal suspension and wait 30 min.
5. After 30 min, swirl the suspension to distribute the cells evenly in the medium and, with a pipette, fill both sides of hemacytometer. Dead cells will be colored blue.
6. Make a differential count of a total of 100 living and dead cells at 400 x magnification over the grids on each side of the hemacytometer This can be done by using a hand talley with four channels, designating one for living cells and one for dead cells on each side of the hemacytometer.
7. Add the living and dead cell counts of each flask and calculate the percentage of dead cells for the triplicate control, carrier control, and toxicant concentrations.
8. Calculate the LC50 by straight-line graphical interpolation as shown in Fig. 3. Plot concentration on the y axis and percentage dead on the x axis.
9. Report counts of living and dead cells in control, carrier control, and all toxicant concentrations, giving counts on each of the three cultures in each of the groups, their averages, and the LC50.

6.1 References

Alexander, L.M. and M. Wilkinson. 1987. A protocol for the validation of vital and mortal stains. Bot. Mar. 30: 109-113.

Crippen, R.W. and J.L. Perrier. 1974. The use of neutral red and Evans blue for live-dead determinations of marine plankton. Stain Technol. 49: 97-104.

Reynolds, A.E., G.B. Mackiernan, and S.D. Van Valkenburg. 1978. Vital and mortal staining of algae in the presence of chlorine-produced oxidents. Estuaries 1: 192-196.

Walsh, G.E. 1983. Cell death and inhibition of population growth of marine unicellular algae by pesticides. Aquat. Toxicol. 3: 209-214.

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